A NUTRICEUTICAL APPROACH TO INHIBITING H1N1 INFLUENZA INFECTION

BY

LEI LU

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS-Veterinary Pathobiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Adviser:

Professor Mark S. Kuhlenschmidt

ABSTRACT

The disease burden caused by respiratory infections in the human infant is greater than any other disease. Human influenza and respiratory syncytial viruses are two major causes of respiratory infections in infant and young children and often augment the development of subsequent bacterial infections. Prevention and treatment strategies for infant respiratory disease are constrained by the lack of effective vaccines, especially against respiratory viruses, and increasing antibiotic resistance. Breast-feeding is widely reported to help protect infants against infectious disease. Some of this protective activity is clearly due to the immune component of breast milk; however, the non-immune component, especially human milk oligosaccharides (HMO) are also thought contribute to antimicrobial activity. Human milk contains a large variety of oligosaccharides, some of which are present in sufficient concentration to potentially function as receptor decoys for respiratory pathogens. To begin to define which of these human milk oligosaccharides (HMO) may possess antiviral activity, as well as to probe whether such HMO could enhance value as a nutriceutical when added to commercial infant formula, we have developed in vitro virus infectivity inhibition assays to screen selected HMO, as well as infant formulas and human milk for anti-influenza virus (H1N1) activity. The results of these screening assays demonstrated that selected HMO were able to block virus infectivity but only at relatively high HMO concentration. The inhibitory concentration necessary to achieve 50% inhibition of infectivity was ~10 mg/ml for all HMO tested with the exception of 3-fucosyllactose (~5 mg/ml); a concentration likely far

exceeding what would be feasible for use as a nutriceutical component of infant formula. Interestingly, in control experiments using various proprietary infant formula preparations, we discovered some of these formula contained relatively potent endogenous anti-H1N1 activities. One of these, XPE, exhibited dose dependent inhibition of both influenza A (H1N1) and human parainfluenza virus (HPIV-3) infectivity. The XPE inhibitory activity was purified by organic solvent extraction and semi-preparative high-performance thin-layer chromatography. Furthermore, a similar antiviral activity also was extracted and partially purified from human milk. These results indicate that a lipid or a lipid-like component is at least partially responsible for the observed antiviral activity of human milk and certain infant formula. These results also suggest the lipid component of infant formula and human milk (HML), and perhaps other natural products are worthy of further exploration as a source of antiviral nutriceuticals. The use of such nutriceuticals, either alone or in combination with other bioactive natural products such as HMO, for the possible prevention and treatment of viral respiratory infection and disease in the human infant, is discussed.

ACKNOWLEDGEMENTS

There are many people I would like to thank. Most importantly, I would like to thank my advisor, Dr. Mark Kuhlenschmidt for all his patient guidance and support in my graduate study. Without his continuous support, it would have been impossible to reach this. I would like to thank my committee members Dr. Federico Zuckermann and Dr. William Laegreid for their helpful suggestions and valuable feedback.

Dr. Theresa B. Kuhlenschmidt deserves special thanks for her kind guidance and advice in my research.

Thanks to Dr. Mariangela Segre, Paula K Moxley, Linda Rohl and all other administrative staff of the department of pathobiology, for their great supports and patients during my graduate study.

Lastly, I thank my family and friends, they constant support, encouragement, and love helped me pursue my graduate education and to strive to achieve excellence in all that I do.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Respiratory viruses, including influenza virus, are the most common cause of respiratory tract disease in infants and young children and are a major public health problem in this age group. The nursing infant is particularly vulnerable to environmental pathogens due to the immaturity of its immune system. Although some progress has been made toward vaccine development during the past decades, the application of vaccines against influenza virus is hampered by the antigenic drift and antigenic shift seen in influenza viruses. Furthermore, current vaccines against respiratory syncytial virus (RSV) have no significant effect on preventing respiratory syncytial virus disease (Simoes, Tan, Ohlsson, Sales, & Wang, 2001). In addition, antibiotics are often prescribed for patients with lower respiratory tract infections, but the increasing emergence of antimicrobial resistance in bacterial pathogens threatening their continued effectiveness. Effective therapeutic or prophylactic agents without side effect are urgently needed for reducing the respiratory disease burden in infants and young children.

The results of considerable research over many years have demonstrated the benefits of breastfeeding and the antimicrobial effects of human milk (C. E. Isaacs, 2011; McVEAGH & MILLER, 1997; Newburg, 1996). Besides immunological components, there are many non-immunological factors in human milk, which are considered to be anti-infective agents for infants, including oligosaccharides and lipids (Espinosa,

Tamez, & Prieto, 2007; C. E. Isaacs, Thormar, & Pessolano, 1986). It is generally believed that human milk oligosaccharides act as soluble receptor analogs that inhibit the attachment of pathogenic bacteria, viruses, and bacterial toxins to the mucosa of the infant colon and respiratory tract (Urashima et al., 2009). Some reports show that monoglycerides and free fatty acids produced from human milk lipolysis possess strong antiviral activity against enveloped viruses, regardless of whether the virus is a DNA or RNA virus (C. E. Isaacs, 2011). These non-immunological, anti-infective factors are receiving more and more attention because of their potential nutriceutical, nonimmunogenic and nontoxic characteristic.

The overarching hypothesis of the research presented here is that natural, non-immune components of human milk, such as HMO or HML have the potential to act as antiviral nutriceutical compounds that can effectively inhibit respiratory viruses infection and disease. The approach was to develop an in vitro, virus infectivity-blocking assay for use in screening the antiviral activity of various HMO, commercial and proprietary infant formula, and human milk components. In this screening assay, putative antiviral components were incubated with virus, the mixture added to host tissue culture cells, and virus infectivity quantified using an immunocytochemical assay. Components displaying antiviral activity in this screening assay were then partially characterized as to their identity and cellular mechanism of action.

CHAPTER 2: LITERATURE REVIEW

2.1 Respiratory viral disease of the human infant

2.1.1 Introduction

The human disease burden caused by respiratory infection is more serious than from any other disease (Mizgerd, 2006). According the World Health Organization, there is18% mortality for young children below the age of five caused by respiratory disease; diarrheal disease (15%) and malaria (11%) were the next greatest killers among young children (World Health Organization, 2005). Besides the increased mortality, respiratory infection also accounts for increased morbidity in young children. Clinical reports indicate 22%of all children hospitalizations (Nicholson et al., 2006) and 59% of general consultations (Olszewska, Zambon, & Openshaw, 2002) are the result of respiratory viral infection. Respiratory tract infections in the young also cause a large burden on national healthcare budgets (Nicholson et al., 2006). Every year 3% of all children less than 1 year old are admitted to the hospital with respiratory infections (D. K. Shay, Holman, Newman, Liu, Stout, & Anderson, 1999) and of these, viruses are the most common cause of both upper and lower respiratory tract disease in infants and young children.

2.1.2 Infectious agents

In temperate climates, most respiratory viruses infections usually occur in the winter. Disease peaks occur in January and February in the United States. Acute respiratory

tract infections, especially those resulting from influenza virus, respiratory syncytial virus and human parainfluenza virus contribute to a significant portion of medically attended illness and hospitalization of children younger than 5 years old (Figueras-Aloy, Carbonell-Estrany, Quero, & IRIS, 2004; Harper et al., 2009). It is worth pointing out those complications secondary to viral-induced respiratory infection, such as otitis media, pulmonary disease, and secondary bacterial infections are not limited to high-risk children but also frequently affect otherwise healthy children (C. B. Hall et al., 2009; Mullins et al., 2004). Evidence provided by studies in the United States indicate more than 152 influenza-related deaths in children occurred during the 2003-2004 influenza season, 47% of these children were previously healthy (Greenberg & Piedra, 2004).

Respiratory syncytial virus is known as the primary causative agent of hospitalization in the first year of life for children in the world, and approximately100% of children in the United States are infected with the virus by age three. During the winter, RSV can infect up to 80% children less than two years of age (Leader & Kohlhase, 2002; Robert C., 1998; D. K. Shay, Holman, Newman, Liu, Stout, & Anderson, 1999c; Thompson et al., 2003). The disease caused by RSV infections is the most severe in premature infants and RSV has been reported to be the leading viral cause of infant death in this group (Thompson et al., 2003). Infants admitted to the hospital because of RSV infection usually need oxygen supplementation, mechanical ventilation and intensive care. There are several distinct disease syndromes that are associated with

RSV infections in children, including bronchiolitis, sudden infant death syndrome, childhood asthma, and post infection wheezing. Currently, there is no licensed available vaccine for RSV prevention (Hull, Thomson, & Kwiatkowski, 2000; Kneyber et al., 1998).

The results of recent research have suggested that influenza virus infection in childhood also causes substantial medical and economic burden due to hospitalizations, medical visits, and antibiotic prescriptions in otherwise healthy children, especially those less than two years old. Although RSV frequently causes more severe disease symptoms, the highest incidence of infection is caused by influenza virus (A. S. Monto, 2002). Although school age children who are infected with influenza virus usually do not require hospitalization, the high incidence of infection in these children results in them being the primary source of infection in a community. In children up to 4 years of age, the hospitalization rate is approximately 100 per 100,000 children without high-risk medical conditions and approximately 500 per 100,000 children with high-risk conditions. The morbidity and economic burden caused by influenza disease is evidenced by the fact that more than 25 million physician visits every year are caused by influenza viruses resulting in direct medical costs in the United States ranging from $1 to 3 billion (Couch, 2000; Patriarca, 1999). Complications of influenza in children include: acute otitis media, sinusitis, bronchitis, pneumonia, encephalitis, myositis, myocarditis, febrile seizures, encephalopathy and Reye's syndrome. As stated previously, school age children are the primary

transmission vectors in influenza epidemics, thus they are the main targets for immunization to prevent influenza spread in the general population (A. S. Monto & Sullivan, 1993; Andres, Donovan, Kuhlenschmidt, & Kuhlenschmidt, 2007)

Other respiratory viruses contributing to the respiratory disease burden in children are the human metapneumoviruses and parainfluenza viruses. Human metapneumovirusesare responsible for 5-6% of respiratory disease in hospitalized children less than 5 years of age (van den Hoogen, Osterhaus, & Fouchier, 2004), whereas the parainfluenza viruses are second only to RSV increasing hospitalizations for lower respiratory tract infection in children less than 18 years old (Williams et al., 2004). The clinical symptoms caused by the metapneumoviruses and parainfluenza viruses are similar to RSV (Williams et al., 2004).

Adenoviruses are recognized as another important cause of acute respiratory infections in children, and are responsible for about 3-5% of the lower respiratory tract illnesses (Glezen et al., 1971). An adenovirus vaccine is available for military personnel, but it is not licensed for use in the general population. Other viruses, such as enteroviruses and rhinoviruses, are also responsible for the overall burden of respiratory disease during childhood (Greenberg & Piedra, 2004). Coronaviruses are believed as the cause of severe acute respiratory syndrome (SARS), which was first recognized in Hong Kong during the 2003 epidemic (Lee et al., 2003). SARS morbidity is higher than other respiratory viral disease, approximately one-fourth of

patients need intensive care and ventilation support. Compared to adults, SARS morbidity and mortality appear to be less severe in children. Mortality caused by SARS may range from 3 to 7% with good supportive care. Vaccines for SARS are in development but currently not available (Greenberg & Piedra, 2004).

2.1.3 Upper respiratory tract infection

Most respiratory virus infections in early childhood are limited to the upper respiratory tract, resulting in symptoms of the common cold, such as coryza, cough, and nasal congestion. Rhinitis and pharyngitis also are found frequently associated with upper respiratory infections as is conjunctivitis and tympanic vascular lesions. In some cases, otitis media occurs causing earache, tenderness of the tragus upon pressure, and a red bulging tympanic membrane upon inspection. Upper airway viral infection in infants is also often accompanied by fever and may cause lethargy or irritability and poor feeding. Symptoms caused by upper respiratory infection are usually mild, so specific treatment is usually not required. Antipyretics and nasal decongestants are often used to make feeding easier and reduce discomfort.

2.1.4 Lower respiratory tract infection

Approximately 33% of infants infected with respiratory virus can develop lower respiratory tract symptoms, including wheeze, severe cough, tachypnea, breathlessness, and respiratory distress. These symptoms cause clinical signs such as grunting, nasal flaring, and intercostal retractions, and rarely cyanosis. Auscultation of the chest reveals wheezes, crackles, crepitation, and inspiratory rhonchi or generally

reduced breath sounds that are caused by air trapping and peripheral hyperinflation of the lung (Tregoning & Schwarze, 2010). It is worth emphasizing that the recurrent episodes of apnea in RSV infection area serious threat to infants less than 6 months old (Darville & Yamauchi, 1998).

Distinguishing between bronchitis, bronchiolitis, and pneumonia to define predominantly proximal airway disease, conducting airway disease, or involvement of the alveolar compartment, respectively, is a frequent method often used to classify respiratory illness. The use of these classifications to make clinical diagnoses is controversial and lacks international acceptance, especially since the treatment is the much the same regardless of the classification (Tregoning & Schwarze, 2010). The strategy for treatment of lower respiratory tract infections (LRTI) are based on evaluation of the severity of respiratory damage by measuring the O_2 saturation, blood gas values, and clinical evaluation of the severity of the respiratory distress (Adcock, Stout, Hauck, & Marshall, 1997). Other risk factors (e.g., crowding, gender, nutrition) and preexisting illness (e.g., chronic lung disease associated with prematurity) may also increase the severity of respiratory infections and thus influence strategies for disease management (Pavia, 2011).

Extra pulmonary manifestations of LRTI in RSV infections are rarely observed. These manifestations include seizures, hyponatremia, cardiac arrhythmias, cardiac failure, and hepatitis (Eisenhut, 2006). While the epithelium is the primary site of infection

and viral replication, viral RNA not only has been found in the respiratory tract but also been transiently detected in peripheral blood mononuclear cells and, very rarely, in cardiac muscle and cerebrospinal fluid (Eisenhut, 2006).

2.1.5 Bacterial co-infection

An often-serious complication of respiratory viral illness is increased susceptibility to bacterial subsequent infection. This is most often seen in influenza but also has been reported for RSV infection (Rothberg, Haessler, & Brown, 2008a; Valarcher et al., 2001). Evidences show that many of the deaths in the 1918-1919flu pandemic were due to secondary bacterial pneumonia (Klugman & Madhi, 2007).Viral infection facilitates bacterial infection through two mechanisms: compromising host physical barriers and altering the immune response. Studies have shown that viral infection and subsequent immune response may alter the respiratory epithelia, enhancing bacterial colonization and entry (Plotkowski, Puchelle, Beck, Jacquot, & Hannoun, 1986). For example, influenza virus can thin mucus and expose receptors on epithelial cells by the action of viral neuraminidase activity that is exploited for bacterial adhesion (Peltola, Murti, & McCullers, 2005). In addition, viral infection can suppress the innate immune response. Studies have shown that influenza virus infection can inhibit the initial neutrophil response thus increasing the risk for subsequent bacterial infection (Colamussi, White, Crouch, & Hartshorn, 1999; McCullers, Iverson, McKeon, & Murray, 2008; McNamee & Harmsen, 2006; van der Sluijs et al., 2006). Usually, bacterial co-infection occurs in the later stages of viral infection, after

dampening of the immune response has occurred. A crucial cytokine, IL-10, important in the resolution of the immune response, is increased in viral respiratory infections and may facilitate early dampening of the immune response and facilitate subsequent bacterial infection (van der Sluijs et al., 2004). A general down regulation of the host's ability to detect pathogens, which is thought to happen following viral infection, also may increase the risk of intercurrent bacterial infection (Didierlaurent et al., 2008). As stated earlier, the incidence and consequence of bacterial co-infections in viral respiratory disease have resulted in significant increase in the use of prescription of antibiotics (Low, 2008).

2.2 Influenza Virus

Influenza viruses are a major cause of annual flu epidemics worldwide. In a typical year, approximately 15% of the U.S. population can be infected, leading to about 36,000 deaths and more than 200,000 hospitalizations (Molinari et al., 2007). Influenza A viruses are enveloped, negative-sense, single-stranded RNA viruses of the *Orthomyxoviridae* family (Steinhauer & Skehel, 2002). The shape of influenza viruses is somewhat variable: ranging from spherical particles to more elongated oval forms, of 80–120 nm in diameter. Their genome contains eight RNA segments that encode for eleven viral proteins (Tumpey et al., 2004). Three of these proteins are present on the surface envelope of influenza viruses: hemagglutinin (HA), neuraminidase (NA), and matrix protein (M1). Other encoded proteins include nucleoprotein (NP), RNA polymerase subunits (PB1, PB2, PA), a pro-apoptotic PB1-F2 protein, a proton-selective ion channel protein (M2) and nonstructural proteins

(NS1 and NS2) (Chen & Lander, 2001). Among these proteins, HA and NA are the antigenic determinants of Influenza A viruses and the basis for their classification into different serotypes.

Sixteen HA and nine NA serotypes have been identified thus far (Viswanathan et al., 2010). The lack of a proofreading mechanism in Influenza virus's polymerase leads to an error-prone process of transcription during virus. The selection and accumulation of these altered transcripts (mutations) is responsible for development of new Influenza A virus serotypes. This process, known as antigenic drift, helps the virus evade the host immune system by changing the HA, NA and M1 antigens exposed on the surface envelope. Furthermore, the segmented characteristic of viral RNA permits reassortment of genes between viruses of different serotypes and creation of a new virus subtypes. This process is known as antigenic shift. Both antigenic drift and antigenic shift are the principal mechanisms driving the evolution of influenza viruses (Nelson & Holmes, 2007). Antigenic drift is a continuous process and leads to gradual changes in surface antigens, while antigenic shift is an occasional occurrence and causes more immediate and significant changes in the virus antigenicity.

2.2.1 Pathophysiology of Influenza virus

Initial infection of respiratory tract with influenza viruses is likely due to direct nasal inoculation or inhalation of small particle-aerosols containing virus. Studies in volunteers have shown that virus delivered through aerosol route is at least 100-fold

more infectious than administrated by nasal drops (O'Donoghue, Ray, Terry, & Beaty, 1973). It is still not well understand which mode of infection is predominant in nature. After initial infection, there is widespread involvement of the trachea, bronchi, and lower respiratory tract. Bronchoscopy with patients infected with typical, uncomplicated (non-pneumonic) acute influenza found diffuse inflammation of the larynx, trachea, and bronchi with mucosal injection and edema (Brady et al., 1990). Histological examination in these cases has revealed a range of histological findings from vacuolization of columnar cells with cell lose to extensive desquamation down to the basal layer of cells. Viral antigen can be detected in epithelial cells but is not found in the basal cell layer (Galbraith, Oxford, Schild, & Watson, 1969a; Galbraith, Oxford, Schild, & Watson, 1969).

In general, the tissue response becomes more notable as one moves distally in the airway (Reuman et al., 1989). Epithelial compromise usually takes place with cellular infiltrates mainly composed of histiocytes and lymphocytes (Reuman et al., 1989). More severe cases investigated by autopsy show extensive necrotizing tracheobronchitis with ulceration and sloughing of the bronchial mucosa (Clover et al., 1986; Galbraith, Oxford, Schild, & Watson, 1969). Recovery is associated by rapid regeneration of the epithelial cell layer and pseudometaplasia.

Abnormal pulmonary functions are often seen in otherwise healthy, non-asthmatic young adults with uncomplicated acute influenza (Longini, Koopman, Monto, & Fox,

1982; Wingfield, Pollack, & Grunert, 1969). These pulmonary defects include diminished forced-flow rates; flow rates consistent with generalized increased resistance in airways less than 2 mm in diameter. Increased responses to bronchoprovocation (a diagnosis test of asthma) and increases in the alveolar-arterial oxygen gradient also have been seen (Knight, Fedson, Baldini, Douglas, & Couch, 1970; Togo et al., 1970). Furthermore, pulmonary function defects can remain for several weeks after clinical recovery. Influenza in patients with chronic obstructive pulmonary disease or asthmatics may lead to acute declines in forced expiratory volume in 1 sec (FEV1) or forced vital capacity (FVC). Although, primary viral pneumonia is rarely seen as a severe complication of acute influenza (DIEFENBACH & WINTER, 1958; Rothberg, Haessler, & Brown, 2008), these patients have increased susceptibility to bronchoconstriction from air pollutants (e.g., nitrates) (Younkin, Betts, Roth, & Douglas, 1983). In patients where, viral infection of the lung has occurred, it is most often due to contiguous spread from the upper respiratory tract. In these patients, the disease is universally severe and is manifested by tracheitis, bronchitis and bronchiolitis as well as bloody fluid in the trachea and bronchi, hyperemic mucosa, submucosal hyperemia, focal hemorrhage, edema, and cellular infiltrate as well as loss of normal ciliated epithelia cells (Rothberg, Haessler, & Brown, 2008). In addition, varying numbers neutrophils and mononuclear cells mixed with fibrin and edema fluid present in the alveolar spaces and the alveolar capillaries are often remarkable hyperemic with intra-alveolar ducts and alveoli (Little, Hall, Douglas, Hyde, & Speers, 1976).

2.2.2 Influenza virus infection cycle

The infection cycle of influenza A virus starts with the attachment of the virus to sialic acid residues present at the non-reducing termini of oligosaccharide chains of N-linked glycoprotein receptors on the host cell surface. The attached virus is internalized through endocytosis, followed by membrane fusion and uncoating of the virus envelope. Following entry into the host cell, the viral RNA is transported to the nucleus to undergo replication and transcription. Newly synthesized proteins and viral RNA are eventually transported to the host cell surface membrane to form new virions. In this last stage of the virus infection cycle, the new infectious virions bud out of the host cell membrane with HA and NA present on the newly formed virus envelope that is derived from the host cell surface membrane (Viswanathan et al., 2010).

2.2.3 Glycoconjugates of the host cell surface

Glycoconjugates represent a major network of informational molecules on the cell surface and extracellular matrix (ECM) of epithelial cells (Frevert & Sannes, 2005). Glycans on the cell surface are covalently attached to proteins or lipids to form glycoconjugates, which can be linear or branched in structure and participate in a number of diverse and complex cellular processes involved in regulating how the cell recognizes and interacts with its microenvironment. Glycoconjugates represent a vast repertoire of dynamic molecular antenna. These antenna, composed primarily of extended chains of complex carbohydrate (oligosaccharide) moieties are used by the

cell to initially regulate cell-cell and cell-ECM adhesion and resultant signal transduction pathways involved in such complex and diverse processes as embryogenesis, cancer metastasis, and response to infectious disease pathogens (Frevert & Sannes, 2005). Numerous studies have shown glycoconjugate oligosaccharides play an important role in microbial pathogenesis and invasion. Many microbial pathogens exploit host cell surface glycans as attachment sites or receptors that increase their ability to infect and invade host epithelia (Bernfield et al., 1999). The binding of pathogens to glycan receptors on host cells (microbial adhesion) is accomplished by microbial surface proteins recognizing specific host cell carbohydrate sequences or conformational domains. Accordingly, there is considerable interest in defining the oligosaccharide epitopes responsible for the microbial adhesion of numerous microbial pathogens in an effort to develop therapeutic receptor mimetics for the non-antibiotic prophylaxis or treatment of infectious disease. Proof of concept for this approach has been provided by a recent study by Bergner et al (Bergner, Kuhlenschmidt, Hanafin, Firkins, & Kuhlenschmidt, 2011).

2.2.4 Human glycan receptors for influenza virus infection

The human upper respiratory tract is the primary site of influenza A virus binding and the glycans terminated with sialic acid (SA) on the epithelial cell surface are receptors for this virus (Ge & Wang, 2011). Attachment of influenza A virus to host cells is initiated through a binding domain on the HA which recognizes SA containing

glycans on the surface of epithelial cell (Y. Suzuki, 2005). The amino acid residues that form the binding domain are determinants for receptor specificity of H1, H2 and H3 viruses. The SAs are normally classified into 2 major groups, *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc), based on the modification of the C-5 amino group (T. Suzuki et al., 1997). The terminal sialic acids of glycans on cell surfaces are linked to galactose by either an α2,3 linkage (α2,3Gal) or an α2,6 linkage (α2,6Gal) and the distribution of these linkages as well as the specific types of SA (N-acetyl or N-glycolyl) expressed on cell surfaces varies according to animal species. For example, bovine, equine and swine tissues possess both NeuAc and NeuGc, whereas human tissues possess only slight concentrations of NeuGc (less than 0.1% of total SA) (Y. Suzuki et al., 2000). Influenza A viruses also differ in their recognition of NeuAc, NeuGc (Higa, Rogers, & Paulson, 1985). Most influenza A viruses isolated from avian preferentially recognize NeuAcα2,3Gal, whereas most human isolates preferentially recognize NeuAcα2,6Gal (Rogers & Paulson, 1983). This distinct receptor preference is due to specific amino acid differences in HA. For H1 viruses, avian virus isolates have a glutamate at position 190 (Glu-190) in the HA, but for human isolates position 190 is an aspartate (Asp-190) residue. Thus, Glu or Asp located at position 190 in the HA of influenza viruses determines preferential binding to α2,3 or α2,6 linkages, respectively (Kobasa et al., 2004; Matrosovich et al., 2000; Stevens et al., 2004). In avian viruses, Gln at position 226 in HA (Gln-226) correlates with preferential binding to SAα2, 3Gal, whereas Leu-226 in human H2 and H3 viruses determines HA specificity for SAα2,6Gal (Rogers & Paulson, 1983;

Matrosovich et al., 2000). In most human isolates, Leu-226 is accompanied with Ser-228, whereas Gln-226 is accompanied with Gly-228 in avian viruses. NeuGcα2, 3Gal recognition has been found to be critical for viral infection and replication in ducks (T. Ito & Kawaoka, 2000) and horses (Y. Suzuki et al., 2000). Interestingly, swine influenza viruses bind equally well to both NeuAcα2,6Gal and NeuAcα2, 3Gal (T. Ito et al., 1997; T. Suzuki et al., 1997). In addition, in the human respiratory tract the predominant receptors on epithelial cells are SAα2,6Gal-terminated glycans, whereas SAα2,3Gal-terminated glycans predominate on intestinal epithelial cells of ducks (T. Ito et al., 1997; Y. Suzuki, 2005). Combined with the virus binding preferences described above, a hypothesis has been proposed: the type of linkage of SA molecules to galactose on the host cell surface is responsible for the host range restrictions of influenza viruses. However, both human (α2,6) and avian (α2,3) receptors have been found in pig trachea (T. Ito et al., 1998;Y. Suzuki et al., 2000), in the intestine and trachea of quail (Wan & Perez, 2006) and the respiratory tract of humans (Shinya et al., 2006). These relatively recent findings suggest the host restriction of influenza A viruses is dynamic and dependent on host exposure.

2.2.5 Mixing vessel hypothesis

In 1985, Scholtissek et al., who proved that swine infected with swine influenza virus also could be dually infected with human or avian influenza A virus, proposed the "mixing vessel" hypothesis (Scholtissek, Bürger, Kistner, & Shortridge, 1985). Such a dual infection may produce viral reassortants containing swine, human, and/or avian

genes by antigenic shift. Sometimes, this combination of swine, human and avian genes may produce a human pandemic virus by introduce unique virus serotypes into the human population. The “mixing vessel hypothesis” was formulated based on the genetic similarities between certain subtypes of swine, human and avian influenza viruses and the susceptibility of swine to infection by both avian and human viruses (Ma et al., 2009).

In support of this hypothesis is the receptor specificity of avian and human influenza viruses. As stated above, avian viruses preferentially bind to SAα2,3Gal-terminated oligosaccharides while human viruses incline to bind SAα2,6Gal-terminated oligosaccharides, and these receptor types are differentially expressed in avian and human epithelial cell surfaces. In contrast, both receptor types can be expressed on the respiratory tract of swine (T. Ito et al., 1998). So Swine are susceptible to both human and avian viruses. Thus, virus binding to specific terminal sialic acid linkages on host cell surface glycoconjugates is not only a required step for virus infectivity, but also for controlling tissue tropism and host range. Therefore, blocking virus binding by using exogenously added, for example in human infant formula, natural oligosaccharides as receptor competitors may be an attractive approach for developing antiviral nutriceuticals. Such nutriceuticals may prove extremely useful in situations of vaccine failure or vaccine unavailability.

2.3 Respiratory syncytial virus

2.3.1 Introduction

Respiratory syncytial virus (RSV), first characterized in 1957, has long been considered as the most important cause of acute lower respiratory tract infection in infants and young children worldwide (C. Hall, 1999). RSV is highly contagious, approximately 70% of young children will acquire RSV infection during the first year of life (B. S. Graham, Johnson, & Peebles, 2000). When children are infected with RSV, most of them suffer from mild upper respiratory tract infection, but approximately 40% of these infections will develop lower respiratory infections (D. K. Shay, Holman, Newman, Liu, Stout, & Anderson, 1999). In immune compromised patients, RSV infection can cause respiratory failure resulting in a high (up to 70%) mortality rates (Hertz, Englund, Snover, Bitterman, & McGlave, 1989). Unfortunately, there is no effective RSV vaccine available.

2.3.2 Virology

RSV is a member of Paramyxoviridae family of ribonucleic acid viruses. RSV was first isolated in 1956 from a colony of chimpanzees with coryza and designated chimpanzee coryza agent. Shortly thereafter it was identified from an infant with bronchitis. Negative staining electron microscopy reveals RSV virions are pleomorphic and have a nucleocapsid enclosed by lipid envelopes with a diameter ranging from 150-300 mm (Chanock, R.M 1957). The RSV genome is a single-stranded negative-sense RNA and encodes 11 proteins: non-structural (NS2, NS1),

nucleocapsid (N),phosphoprotein (P), matrix (M1), small envelope (M2), small hydrophobic (SH), RNA-dependent RNA polymerase (L), surface fusion glycoprotein (F) and surface attachment glycoprotein (G) (Hacking & Hull, 2002). The lipid envelope surrounding the nucleocapsid is host-derived as a result of budding from the host cell surface, and contains three transmembrane glycoproteins encoded by virus: F, G, and SH. The NS1 and NS2 nonstructural proteins appear to preferentially inhibit IFN-α/β signaling by down regulation of Stat2 transcription activator expression (Lo, Brazas, & Holtzman, 2005). The G glycoprotein functions in attachment of RSV to the surface of host respiratory epithelial cells and variation in its carbohydrate epitope defines the antigenic determinant of the A and B subgroups of RSV (Peret, Hall, Schnabel, Golub, & Anderson, 1998). Both subgroups often circulate simultaneously within geographically confined epidemics, although group A is dominant (Falsey & Walsh, 2000). Currently, the relationship between RSV strain variation and pathogenesis is not well understood. Some results indicate more severe disease is seen in children with group A viruses infections (Falsey & Walsh, 2000), whereas many other studies suggest there is no differences in clinical severity between virus subgroups (Brandenburg, van Beek, Moll, Osterhaus, & Claas, 2000; Falsey & Walsh, 2000).

2.3.3 RSV infection

RSV exposure by direct aerosol contact with the nose or eyes leads to viral infection in the nasopharynx within 4 to 5 days post-exposure and occasional spread to the

lower respiratory tract (C. B. Hall, 2001). Clinical signs of upper respiratory tract infection are common and include rhinorrhea, cough, and low-grade fever, whereas lower respiratory tract infections usually present as bronchiolitis and associated wheezing, air trapping, and increased airway resistance (Collins & Graham, 2008).Pneumonia also is a frequent complication seen in RSV-infected infants (C. B. Hall, 2001; McNamara & Smyth, 2002). Infection of the upper respiratory tract usually is limited to the most superficial cells of the respiratory epithelium (J. E. Johnson, Gonzales, Olson, Wright, & Graham, 2006). In the lower respiratory tract, the major targets of RSV are the ciliated cells of small bronchioles and type 1 pneumocytes located in the alveoli. Other cells, including non-ciliated epithelium and intra-epithelial dendritic cells (DCs) may also be infected, but basal cells are usually spared (J. E. Johnson et al., 2006).

The cellular receptor for RSV has not been unequivocally identified, but in vitro studies provide some clues about the mechanisms of RSV entry. Heparin-like glycosaminoglycans, unbranched polysaccharide chains on the surface of most mammalian cells have been shown as receptors or co-receptors for RSV (Mart ínez & Melero, 2000) or are involved with virus entry (Hallak, Collins, Knudson, & Peeples, 2000). Although glycosaminoglycans are believed to be important for RSV G protein interaction with target cells, the precise cellular receptor of RSV is still unknown. The RSV F protein, which mediates viral penetration by fusion of the viral envelope with the host cell plasma membrane, has been shown to bind to intercellular adhesion

molecule-1 (ICAM-1) (Behera et al., 2001; Kurt-Jones et al., 2000; Malhotra et al., 2003). In addition, electron microscopy studies have shown the virus G protein, and the host-cell-derived lipid, GM1, are mainly distributed in virus filaments (Jeffree et al., 2007). Further, lipid raft domains have been implicated in the assembly of new virus particles (Brown et al., 2004; Jeffree, Rixon, Brown, Aitken, & Sugrue, 2003).

2.3.4 Pathogenesis of RSV disease

The mechanism(s) of RSV pathogenesis is not well understood. The relative contribution of viral versus various host factors remains controversial (DeVincenzo, 2005; Groothuis, Hoopes, & Jessie, 2011). RSV pathogenesis is affected by a number of host factors, such as the age of infection, genetic predisposition, and host immune response (DeVincenzo, 2005). Additionally, the state of immune maturation early in life is also an important determinant for RSV infection. RSV-specific maternal serum antibodies have been shown to suppress antibody and T-cell responses to primary RSV infection (DeVincenzo, 2005).

While details of molecular mechanisms underlying RSV disease are not well understood, abundant evidence indicates the early inmate host response to primary infection is important. Overly robust inflammatory responses strongly correlated with the severity of RSV disease. For example, several studies have demonstrated that RSV could exacerbate asthma and bronchiolitis (Sikkel, Quint, Mallia, Wedzicha, & Johnston, 2008), and that these conditions are accompanied by inappropriate cytokine

expression, enhanced CD4 T-cell responses, inflammation and reduced immune regulation (Kallal & Lukacs, 2008; Sikkel et al., 2008). A large variety of inflammatory mediators are produced in the response to RSV infection, and there is controversy as to their relative importance (Oshansky, Zhang, Moore, & Tripp, 2009): Th2-type biased immune response caused by some RSV antigens have been supported by considerable evidence (Becker, 2006b; B. S. Graham, Johnson, & Peebles, 2000a; MARTINEZ, 2003; van Schaik, Welliver, & Kimpen, 2000), but other studies have indicated Th1-biased response or mixed responses associated with severe pediatric RSV infection (Brandenburg et al., 2000; Castro et al., 2008; Garofalo et al., 2001).

The fact that RSV infects infants very early in life suggests that features modulating innate immunity play an important role in the disease process, and it is possible these features are related to RSV activation of pathogen recognition receptors (PRR) or toll-like receptors (TLR) (Pasare & Medzhitov, 2004). How RSV recognition and the subsequent immune responses are regulated by the individual PRRs or TLRs remains unclear. Some studies suggest surface and cytoplasmic TLR including TLR2, TLR3, TLR4 and RIG-I may recognize RSV (Groskreutz et al., 2006; Groskreutz et al., 2006; Haynes et al., 2001; Murawski et al., 2009). Studies show that RSV can modify the expression patterns of cytokine and chemokine expression during infection (Groskreutz et al., 2006; B. S. Graham, Johnson, & Peebles, 2000), and may result in immune deregulation.

The quality of adaptive immunity to RSV infection is significantly influenced by the

interface with the innate immune response. Inappropriate innate and inflammatory responses caused by RSV disease may lead to inappropriate T-cell responses. There are considerable evidences of a Th2-type biased immune response specific for some RSV antigens (Becker, 2006a; B. S. Graham, Johnson, & Peebles, 2000d).

In summary, there is no single or typical pattern for how the cascade of inflammatory mediators affects RSV disease pathogenesis. RSV pathogenesis is the result of multiple factors including virus replication, and aberrant innate and adaptive immune responses.

RSV disease in children

RSV has been primarily known as young children's virus. RSV is associated with50%-90% of cases of bronchiolitis and 5%-40% of cases of pneumonia in young children (Miller, 2010). The incidence of RSV in children is significant. It is estimated that 55,000 to 125,000 RSV associated pediatric hospitalizations and has a mortality rate of about 2% in the United States annually (Cooper, Banasiak, & Allen, 2003; Susan, 2010; C. B. Hall et al., 2009; D. K. Shay, Holman, Roosevelt, Clarke, & Anderson, 2001). Some studies suggested that there is an association between early, severe RSV infection, and recurrent wheezing or asthma in later childhood (Langley & Anderson, 2011; Sigurs et al., 2005). However, whether RSV infection sets up a process that leads to recurrent wheezing or, alternatively, severe disease in children already predisposed to recurrent wheezing has yet to be determined (Korppi, Piippo-Savolainen, Korhonen, & Remes, 2004; Sigurs et al., 2005).

2.4 Human milk oligosaccharides and lipids as inhibitors

2.4.1 Introduction

Breastfeeding is considered to be one of the most cost-effective community health practices for the prevention of infant morbidity and mortality caused by infectious disease (Black, Morris, & Bryce, 2003). Additionally, human milk is not only widely recognized as the ideal food for young infants, it also is considered the model "nutriceutical", or food contributing nutritional or health benefits (Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005). Human milk has been reported to provide protection from respiratory tract infections, diarrheal diseases, bacteremia and meningitis (Hamosh, 2001; Morrow & Rangel, 2004). This protection afforded to breastfed infants is thought to be due to a variety of complementary components, such as colostrum antibodies, and probiotics, found in human milk. Besides milk antibodies, there are many non-immunological factors in human milk, which are considered to have antimicrobial activity, including oligosaccharides and lipids (Espinosa et al., 2007; C. E. Isaacs, Thormar, & Pessolano, 1986).

2.4.2 Structure of human milk oligosaccharides

Among the forms of carbohydrate in human milk, such as lactose, glycolipids, glycoproteins, mucins, glycosaminoglycans, and oligosaccharides, the oligosaccharides are present in the greatest abundance. One liter of human milk

includes approximately 5-10 g of unbound oligosaccharides, which comprises the third largest solid component in milk, after lactose and triglyceride (Bode, 2009). Human milk oligosaccharides (HMO) consist of combinations of five different monosaccharides: D-glucose (Glc3), D-galactose (Gal), N-acetyl-glucosamine (GlcNac), L-Fucose (Fuc), and sialic acid (NeuAc) (McVeagh & Miller, 1997). In most milk oligosaccharides, lactose (Galβ1-4Glc) forms the reducing end, and fucose and/or sialic acid forms the non-reducing terminus. Lactose can be sialylated in α2,3 and/or α2,6 linkages to form 3′sialyllactose and 6′sialyllactose, respectively (C. Kunz, Rudloff, Baier, Klein, & Strobel, 2000). In addition, lactose can also be fucosylated in α1,2 and α1,3 linkages to form 2′-fucosyllactose and 3-fucosyllactose, respectively (McVeagh & Miller, 1997; Newburg, 1996). These trisaccharides are recognized as the short-chain milk oligosaccharides. More complex milk oligosaccharides are formed through the elongation of lactose with up to 15 N-acetyl lactosamine repeats units (Galβ1-3/4GlcNAc). In the complex oligosaccharides, lactose or the polylactosamine backbone can be fucosylated in α1,2, α1,3, and/or α1,4 linkages and/or sialylated in α2,3 and/or α2,6 linkages. At present, approximately 200 different complex oligosaccharides have been identified in pooled human milk (Ninonuevo et al., 2006).

2.4.3 Oligosaccharides as soluble receptor decoys for pathogenic bacteria and viruses

For many years, milk oligosaccharides were thought to only contribute to the development of a specific intestinal flora in infants. Today, there is increasing

evidence that oligosaccharides in human milk may also function as soluble receptor analogues or decoys of epithelia cell-surface carbohydrate receptors (C. Kunz, Rudloff, Baier, Klein, & Strobel, 2000). Because milk oligosaccharides survive gastric acid hydrolysis and digestion/fermentation in the small intestine, their antimicrobial activity mostly depends on their structural configuration (Espinosa et al., 2007). Thus, their specificity and affinity for competing with epithelial cells to impeding pathogens are determined by their structure (Sharon & Ofek, 2000).

Pathogen adhesion to the epithelial cell is the prerequisite step for infection. Microbial binding proteins, such as bacterial adhesins, and virus hemagglutinins, recognize carbohydrate termini on host cell surface glycoconjugates. As discussed earlier for influenza, these receptors also determine the tissue tropism and host range of an invading pathogen (Schneider-Schaulies, 2000). Similarly, humans of a given blood group type are more susceptible to certain pathogens because of the presence of particular carbohydrate receptors on their epithelial cell membranes (McVeagh & Miller, 1997). Although many of these carbohydrate receptors exhibit remarkable structural specificity (e.g., α2,6vs.α2,3 sialic acid binding by influenza viruses (Zopf & Roth, 1996)), their binding affinity, especially when added as soluble oligosaccharides is likely too weak to effectively compete for pathogen binding unless they are present in high concentrations similar to that found in human milk or as multivalent aggregates (Bergner, Kuhlenschmidt, Hanafin, Firkins, & Kuhlenschmidt, 2011), such as exists on the milk fat globule membrane (Sánchez-Juanes, Alonso,

Zancada, & Hueso, 2009). Pathogen-binding carbohydrate receptors present on host cell plasma membrane are in the form of multivalent glycoprotein or glycolipid aggregates or domains. Such a configuration greatly increases the binding avidity between pathogen and host cell and thus is frequently resistant to competition by soluble oligosaccharides added in relatively low (<1 mg/ml) concentration (Bergner, Kuhlenschmidt, Hanafin, Firkins, & Kuhlenschmidt, 2011a; M. D. Rolsma, Kuhlenschmidt, Gelberg, & Kuhlenschmidt, 1998).

Currently, there are at least 20 specific human milk oligosaccharides known that could competitively bind to pathogens of the intestinal, respiratory and urinary tract and the number is growing rapidly (Bode, 2009). These pathogens not only include bacteria, viruses but even yeast (*Candida albicans*) (McVeagh & Miller, 1997). For example, the attachment of influenza virus could potentially be blocked by either NeuAc(α2,6)Lac (influenza virus A), NeuAc(α2,3)Lac (influenza virus B) in human milk (W. Weis et al., 1988). Milk oligosaccharides also prevent the adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to pharyngeal or buccalepithelial cells (Rudloff, Pohlentz, Diekmann, Egge, & Kunz, 1996). Sialylated HMO also block the binding of *Escherichia coli* strains that cause neonatal meningitis and sepsis (C. Kunz & Rudloff, 1993). Compared to formula fed infants, these pathogens are found less commonly in the stool of breast fed infants (C. Kunz & Rudloff, 1993). Furthermore, Human milk oligosaccharides also prevent haemagglutination of *E. coli* and *Vibrio cholera* (Newburg, 1996). Hence, human milk

bathes the epithelial cell membrane of the nasopharyngeal, gastrointestinal and urinary tracts of infants with oligosaccharides that may represent an important antimicrobial component that reduces the incidence of infectious disease in the breast fed infant.

2.4.4 Summary

Adhesion to host cells is a prerequisite for the infection of microbial pathogens. Human milk oligosaccharides competitively bind to the pathogen's carbohydrate-binding proteins and thereby facilitate pathogen clearance by the physiology mechanism characteristic of the host tissue, such as mucociliary action in the respiratory tract or bulk fluid movement in the gastrointestinal tract (Ebrahim, 1997). Now that the efficient, large-scale synthesis of some human milk oligosaccharides is possible, it may also be feasible to add bioactive HMO to infant formula to enhance its antimicrobial activity similar to that seen for human breast milk. Since HMO are natural products, they represent effective, safe, non-immunogenic and non-drug alternatives to, or supplements for, antibiotics or vaccines. Furthermore, because oligosaccharides do not act by killing or arresting the growth of the pathogens, resistance to them is less likely to develop.

2.5 Antimicrobial lipids in human milk

2.5.1 Introduction

In addition to their nutritional value, human milk lipids have broad-spectrum

antimicrobial activity. The antimicrobial ability of lipids, in particular fatty acids, has been studied since the late nineteenth century (Kabara, Swieczkowski, Conley, & Truant, 1972a; Kabara, 1980). Lipid-dependent antimicrobial activity in milk is associated with the release of monoglycerides and free fatty acids by lipolysis from milk triglycerides. These triglycerides are present in the form of fat globules and contribute 98% of the milk fat (Jensen, 1996). Monoglycerides and diglycerides are present in trace amounts. The triglyceride core of the milk fat globule is covered by a membrane of polar lipids and forms an organized structure known as the milk fate globule membrane (MFGM). The MFGM protects the triglycerides from hydrolysis by lipases in milk, and then the antimicrobial activity of these lipids is released by the combination of gastric lipases and pancreatic collapse in the infant's gastrointestinal tract (C. E. Isaacs, Kashyap, Heird, & Thormar, 1990). Lipids are one of a number of non-specific and nonimmunologic protective factors existing in human milk which function at mucosal surfaces (Hosea Blewett, Cicalo, Holland, & Field, 2008). Nonspecific protective factors like lipids are thought to prevent or inhibit the establishment, multiplication and spread of invading pathogens in the host (C. E. Isaacs, 2011).

2.5.2 Antimicrobial mechanism of milk lipids

Human milk lipids can inactivate numerous enveloped viruses, including HSV (C. E. Isaacs, Thormar, & Pessolano, 1986), influenza virus (Kohn, Gitelman, & Inbar, 1980), respiratory syncytial virus (Hilmarsson, Traustason, Kristmundsdóttir, &

Thormar, 2007), measles virus, VSV and visna virus (C. E. Isaacs, Thormar, &Pessolano, 1986), mouse mammary tumor virus (N. H. Sarkar, Charney, Dion, & Moore, 1973), dengue virus types 1 to 4 (Falkler, Diwan, & Halstead, 1975), Cytomegalovirus (Welsh, Arsenakis, Coelen, & May, 1979), SemlikiForestvirus (Chirico, Marzollo, Cortinovis, Fonte, & Gasparoni, 2008), Japanese Bencephalitis virus (Fieldsteel, 1974), HIV and simian immune deficiency virus (C. E. Isaacs, Thormar, & Pessolano, 1986c; Li et al., 2009). Human milk lipids also inactivate both Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*) bacteria (C. E. Isaacs, Kashyap, Heird, & Thormar, 1990), as well as the protozoal pathogens, such as *Giardialamblia* and *Trichomonasvaginalis* (Li et al., 2009; Rohrer, Winterhalter, Eckert, & Kohler, 1986) and *Cryptosporidium parvum* (Schmidt & Kuhlenschmidt, 2008).

The antibacterial and antifungal ability of many of these bioactive lipids may be due to a membrane disruption mechanism as shown for enveloped viruses (Kabara, Swieczkowski, Conley, & Truant, 1972). Exposing bacterial to mild heating to increase membrane fluidity has been shown to increase lipid-dependent killing of the Gram-negative bacterial pathogens *E. coli* and *Pseudomonas aeruginosa*by 1,000 to 100,000 fold, providing suggestive evidence that the bacterial membrane is the target of human milk (C. E. Isaacs, 2011). It should be noted, however, the normal mucosal barrier of the intestinal track might protect epithelial cells in the infant's gut from damage following exposure to dietary fatty acids and monoglycerides (C. E. Isaacs,

2011). Although certain lipids are known to have detergent-like membrane solubilizing activity, there are other possible mechanisms, besides a direct effect on the cell membrane, which could explain their antimicrobial effects. For example, earlier studies have shown that unsaturated fatty acids affect cell signal transduction by inhibiting the influx of calcium through the plasma membrane of calcium depleted cells (Gamberucci, Fulceri, & Benedetti, 1997; Ordway, Singer, & Walsh Jr, 1991). Other studies indicate fatty acids inhibit the Fab I enzyme (enoyl-ACP reductase), which catalyzes the final reduction step of the fatty acid elongation cycle in *P. falciparum* (N.M., 2008; Tasdemir et al., 2007).

2.5.3 Summary

The research results summarized above suggest HML and perhaps HMO, especially if presented in the optimal spatial configuration, have potential as relatively inexpensive nutriceuticals to protect or treat infectious disease caused by a rather broad spectrum of microbial pathogens. Accordingly, the focus of the research described in this thesis is to begin to determine 1) which HML or HMO have the greatest antimicrobial effects, 2) their mechanism of action, and 3) whether they can be used singly or in combination as nutriceutical additions to infant formula to more closely simulate the antimicrobial effects of human breast milk.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Unless stated otherwise, commercial lipids and oligosaccharides were obtained from Sigma Chemical Co. and V-Labs, respectively, and were of the highest purity available. Stock oligosaccharide concentrations were made to 100 mg/ml in sterile water and the diluted into the appropriate medium just prior to testing for inhibition of virus infectivity (see Virus Infectivity Inhibition Assay below). Infant formulas (six proprietary formulations composed of different fat and protein system components (see Table 3.1) denoted as: XAD, XPE, XGE, XHE, XCE, XNE) were obtained in either liquid or powder form and stock concentrations of 0.5 -100 mg/ml (w/w) were prepared in sterile water. These stocks were diluted in the appropriate medium just prior to testing for virus inhibition (see below). Silica gel thin-layer chromatography (HPTLC or TLC) plates were obtained from Grace Alltech. Madin-Darby bovine kidney (MDCK) cells were used as the influenza virus (H1N1) host cell line and were obtained from the American Type Culture Collection (ATCC-CCL 22, Rockville, Maryland) and cultured in MEM supplemented with 10% fetal bovine serum (FBS) (Biowest, Miami, FL), and 2.0g sodium bicarbonate (Sigma, St. Louis, MO), and HEPES (Sigma, St. Louis, MO) at 37 C in 5% CO2. Cell suspensions were obtained by harvesting flasks of confluent cells with 0.05% EDTA-trypsin (GIBCO, Carlsbad, CA). Influenza virus A/PR/8/34 (H1N1) were obtained from the American Type Culture Collection (ATCC) (VR 1469, Manassas, VA) and propagated on Madin Darby bovine kidney (MDCK) cells, and culture supernatants were harvested 24 to 72

h post infection (hpi) and stored at -80 C. Primary antibody against Influenza A nucleoprotein (NP) (cat. No. SC80481) and biotin conjugated secondary antibody goat anti-mouse IgG2a-B (cat. No. SC2073, were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Peroxidase staining reagents were as follows: DAB reagent set kit (KPL, Gaithersburg, MD), goat serum (Vector, Burlingame, CA) and ABC kit (Vector, Burlingame, CA).

3.2 Direct virus infectivity inhibition assay

Measurement of the ability of potential inhibitors to directly block virus infection of host cells by inhibiting virus binding was performed as follows. Suspensions of MDCK cells were distributed to each well of 24-well plates (BD, Franklin Lakes, NJ) and incubated in a CO_2 incubator at 37 C to establish monolayers. On the following day, potential inhibitors were serially diluted in MEM without serum in microfuge tubes (Eppendorf, Hauppauge, NY) to obtain an inhibitor concentration range of 0.01 – 250mg/ml. Each inhibitor concentration (150 µl) and control MEM medium samples (150 µl) were combined with an equal volume of H1N1virus (1400 FFU). The microfuge tubes containing the inhibitor-virus mixture were vortexed and incubated in CO_2 incubator at 35 C for 1 h. Following this pre-incubation MDCK cell monolayers in each well of a 24-well tissue culture plate were inoculated with 200 µl of either the pre-incubated control media or inhibitor-H1N1 mixtures for 30 min at 35 C, in 5% CO_2, to allow the virus to adsorb to the cell monolayers. At the end of this incubation the monolayers were washed and incubated in 1.0 ml MEM containing 5% tragacanth gum (Sigma, St. Louis, MO) and 2% FBS at 35 C for 24h. Tragacanth gum

was added to help produce homogeneously sized, easily countable foci in the FFU assay (see Measurement of virus infectivity: focus-forming assay, below) using modifications of a previously described procedure (Dobos, 1976). After several pre-experiments (data not shown), a concentration of 5% tragacanth gum is found to be optimal for producing consistently sized, easily counted foci.

3.3 Indirect virus infectivity inhibition assay

The direct virus infectivity inhibition assay described above was used to screen the ability of putative virus inhibitors to block virus binding to host cells. To determine if any of the inhibitors testing positive in this screening assay could also block virus infectivity at stages in the virus-host cell infectious cycle other than initial virus binding, we developed an indirect virus infectivity inhibition assay. For this assay, potential inhibitors were added before, during, or after virus adsorption, in eight different combinations as shown in the Table 3.2.

Monolayers of MDCK cells in 24-well plates were prepared as described above. Each well of 24-well plates were rinsed twice with 1 ml of PBS, and then varying amounts (0.01 – 250 mg) of inhibitors were diluted in 1.0 ml final volume of MEM plus 2% FBS (MEM plus 2% FBS alone was used as the control) and the plates incubated at 37 C for 5 h. After this incubation, the medium was removed and cell monolayers rinsed twice with PBS. The monolayers were then inoculated with virus alone (1400 FFU) or with virus plus inhibitor (inhibitor-virus mixtures in MEM plus 2% FBS as described above and incubated in a CO_2 incubator at 35 C for 30 min). After virus

adsorption, monolayers were again washed twice in PBS and incubated in MEM-2%FBS containing 5% tragacanth gum with or without inhibitors at 35 C for 24 h as previously described.

3.4 Measurement of virus infectivity: focus-forming assay

The following steps were carried out at room temperature (RT) on a rocker plate unless otherwise noted. The wells were rinsed twice with 1 ml of PBS. The monolayer in each well was fixed for 2 min with 500 µl of 95%ethanol: 10% buffered formalin (1:1). The monolayers were rinsed for 5min with 500 µl of 70% ethanol per well, followed by an additional 5 min rinse with 500 µl of 50% ethanol. The 50% ethanol was removed and replaced with150µl of on 3% H_2O_2 in wash buffer to quench endogenous peroxidase activity and processed for primary and secondary antibody staining to detect H1N1 infected cell foci as previously described (M. D. Rolsma, Kuhlenschmidt, Gelberg, & Kuhlenschmidt, 1998). Stained foci were observed and digital images automatically collected using a Nikon Eclipse TS100 inverted microscope (Nikon) equipped with a software (Metamorph)-controlled motorized stage (Optician, Prior Scientific).

3.5 Organic solvent extraction of XPE

XPE (0.75 g (wet weight=0.776 ml) was transferred to a glass tube and 2.0 ml of methanol followed by 1.0 ml of chloroform were added and mixed well by vortexing. After centrifugation of the extract at 1,000 g for 10 min (Sorvall Superspeed RC2-B, GSA rotor; Kendro laboratory Products, Newtown, Connecticut) the clear, single phase supernatant was transferred into a new glass tube and the pellet re-extracted

with 3 ml of chloroform-methanol-water 4:8:3. Following centrifugation this supernatant was mixed with the previous extract supernatant, evaporated to dryness and stored at 4 C until use. All fractions generated during organic solvent fractionation were compared for antiviral activity based on the original XPE concentration in mg/ml by defining an XPE equivalent concentration. For example, if 0.75 g in of XPE in 1 ml water was used as the starting concentration and the extraction process generated an aqueous and an organic fraction, each of which was evaporated and resuspended in water to 1 ml, then both the aqueous and organic extracts would contain 750 mg/ml equivalents of the original XPE.

3.6 Thin-layer Chromatography

Samples were dissolved in chloroform-methanol (9:1) and appropriate volumes (generally 5 to 20 µl) were applied in 0.5-cm steaks (separated by 0.5-cm blank lanes) on TLC plates (5 x 20 cm). The plates were developed in the chloroform-methanol (9:1) in a short-bed continuous-development chamber. Lipid bands were visualized by primulin spray (Sigma Chemical Co.). Semi-preparative TLC was performed under the same conditions except that analytes (200-300 µl) were streaked across the entire width of multiple silica gel TLC plates (Merck) using a manual TLC Streaker (Alltech). After development, the outer vertical edges of the plates were stained with primulin. These lanes were used as guide for dividing the unstained lanes into horizontal bands that extended across the entire width of the plate. The unstained middle portion of the lipid bands was scraped from the plates and exhaustively extracted with chloroform-methanol (9:1). Silica fines were removed by

centrifugation and filtration. Following evaporation and re-suspension in a volume of solvent equivalent to the original applied volume, appropriate aliquots were tested for inhibitory activity in the virus infectivity inhibition assays.

3.7 Tables

Sample	Protein system (per 100ml)	Fat system (per 100ml)
XGE	1.5g hydrolyzed whey protein	3.61g triglycerides. Oil blend
XPE	1.5g hydrolyzed whey protein	2.17g triglycerides +1.84g predigested fat
XNE	4.3g Intact caseinprotein	None
XHE	4.3g hydrolyzedcasein protein	None
XCE	1.41g protein from NFMD+whey protein concentrate	2.14g triglycerides +1.5g predigested fat(oil blend)
XAD	1.41g protein from condensed milk+whey protein concentrate	3.64g fat(include MG and lecithin)

Table 3.1. Infant formulations composed of different fat and protein system components. Infant formulas were obtained in either liquid or powder form and stock concentrations of 100 mg/ml (w/w) were prepared. NFMD: non-fat milk dehydrate; MG: milk fat globulin.

Column No	1	2	3	4	5	6	7	8
Pretreat MDCK cell 5 h	**Y**	**Y**	**N**	**Y**	**Y**	**N**	**N**	**N**
Inhibitor in adsorption	**Y**	**N**	**Y**	**Y**	**N**	**N**	**Y**	**N**
Inhibitor in medium 24h	**Y**	**Y**	**Y**	**N**	**N**	**Y**	**N**	**N**

Table 3.2. Description of the treatment conditions used in the indirect virus infectivity inhibition assay. Inhibitors were added before, during, or after virus addition to MDCK cells, in eight different combinations. Y: inhibitor added; N: no inhibitor added.

3.8 Statistical analysis

All statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) 16.0. Statistical probability of $P < 0.05$ was considered significant. Univariate analysis of variance was done to compare the effects of various concentrations of different inhibitors on H1N1 infectivity. The effects of different treatment of inhibitors on H1N1 infectivity and virus attachment were analyzed using a one-way analysis of variance (one-way ANOVA) followed by Bonferroni's post hoc test to determine differences between each treatment.

CHAPTER 4: RESULTS

4.1 Validation of FFU assay:

In order to validate the number of focus forming units detected in the focus forming assay was proportional to the concentration of H1N1 virus concentration used to infect MDCK host cells, cell monolayers were exposed to the different H1N1 concentrations. Two-fold serial dilutions of virus stocks were added to MDCK cells for 30 min, the cell layers washed, and incubated at 35 C for 24 h as described in Materials and Methods. Infected cell foci (FFU) were visualized by DAB staining as previously described (M. D. Rolsma et al., 1998), using anti-H1N1 monoclonal antibody (antibody against Influenza A nucleoprotein (NP) from Santa Cruz Biotechnology, Santa Cruz, CA) to detect the presence of H1N1 viral progeny.

Digital images (24/well of a 24-well plate) of infected cell monolayer were captured by automated microscopy and infected cell foci counted as previously described (Andres et al., 2007). As shown in Figure 4.1, a positive linear correlation was observed between the percentage of infected cells and the concentration of virus used to inoculate MDCK cell monolayers.

4.2 Screening of selected HMO for inhibition of virus infectivity

To examine whether human milk oligosaccharides could potentially serve as receptor decoys for H1N1 influenza virus, we selected commercially available oligosaccharides known to be present in relatively high concentration in human milk (C. Kunz, Rudloff, Baier, Klein, & Strobel, 2000), including those with non-reducing

terminal sialic acid, a sugar epitope known to bind type A influenza viruses. The oligosaccharides (0.1 – 10 mg/ml) tested were lacto-N-neotetraose (LNnT), 3'-sialyllactose (3-SL), 6'-sialyllactose (6-SL), 2'-Fucosyllactose (2-FL), Sialic acid (SA) and also the prebiotic Galacto-oligosaccharides (GOS), Fructo-oligosaccharides (FOS). Virus infectivity in the presence and absence of oligosaccharide (0.1 – 10 mg/ml) was scored as the percent of control FFU values obtained in the presence of virus alone. The result of initial screening of HMO inhibitory activity is shown in Figure 4.2.

None of the HMO tested were able to affect 50% inhibition of virus infectivity at concentrations equal to or less than 1 mg/ml. Although, LNnT appeared to inhibit infectivity by 50% at 1 mg/ml the effect was not dose-dependent. In subsequent experiments using a larger concentration range, LNnt displayed an IC_{50} (concentration causing a 50% inhibition of virus infectivity) value $\geq$ 10 mg/ml. Similarly, none of the HMO tested were able to dose-dependently block 50% virus infectivity at concentrations below 10 mg/ml with the exception of 2-FL, 3-FL, 3-SL and SA. Compared to HMO, the prebiotics GOS and FOS displayed almost no ability to inhibit H1N1 (Fig. 4.3). A summary of the IC_{50} values for all putative oligosaccharide inhibitors is shown in Table 4.1.

4.3 Screening of infant formula compounds and human milk for inhibition of virus infectivity

Although the HMO displayed relatively weak antiviral inhibitory activity, it is possible that the addition of selected HMO to infant formula may enhance their

antiviral activity. one plausible mechanism by which this could potentially occur is via adsorption or incorporation of the HMO into fat globules or micelles of the oil components of infant formula. Such an HMO-lipid complex may present the oligosaccharide as a multivalent aggregate thereby increasing its aviditiy for virus sugar receptors. Accordingly, we screened as part of initial control experiments, a numer of different infant formulasand human milk for their ability to inhibit H1N1 activity using the direct virus infectivity inhibition assay. Each compound was tested in duplicate using dilution series comprised of 10 different concentrations. Representative results of a subset (4 concentrations) of these dilution series are shown in Figure 4.4 (see Table 4.2 for a summary of the IC_{50} values determined by analysis of all inhibitor dilution series experiments). These results indicate certain infant formulas exhibited anti-influenza activity in a dose dependent manner at the indicated concentration range. Among these infant formulas, XPE possessed the greatest viral inhibitory activity (IC_{50} = 1 mg/ml, Table 4.2). Except for XPE, and human milk, which have IC_{50} values of 0.98 mg/ml and 1.95 mg/ml respectively, none of the other infant formulas were able to does-dependently inhibit virus infectivity by 50% at concentrations below 2 mg/ml. The order of inhibitory potency was XPE> XCE> XGE. Formulas XNE, XHE, and XAD displayed no antiviral activity. The finding that some infant formulas displayed relatively potent indigenous antiviral activity was somewhat unexpected. Thus, XPE was chosen for further exploration of the mechanism responsible for its ability to inhibit H1N1 infectivity.

The dose-response curves for XPE and human milk are shown in Figure 4.5. The lowest concentration of XPE causing detectable inhibition of virus infectivity was observed between 60 and 240 μg/ml. This was similar to the minimal inhibitory concentration (120 – 480 μg/ml) required for human milk. Furthermore, the concentrations of XPE and human milk causing maximal inhibition of virus infectivity also were in the same range (4-16 mg/ml). Since XPE contains no HMO, these results suggest XPE and human milk may contain similar, non-HMO antiviral components.

4.4 Initial characterization of the mechanism of action of XPE

To begin to characterize the mechanism by which XPE inhibits virus infectivity, we first sought to determine if XPE acts on the virus or on the host cell. Thus, XPE was added before, during, and after the virus adsorption period and the effect on virus infectivity evaluated using the indirect virus infectivity inhibition assay as described in Materials and Methods. The results of these experiments demonstrated that when XPE is present in the cell culture medium, no matter if added before, during, or after virus adsorption, H1N1 infectivity was inhibited (Figure 4.6). The extent of this inhibition varied; however, depending on when XPE was added during the cell infection cycle. The greatest inhibition was seen when XPE was present at all three stages of the infectious cycle (adsorption, entry, and replication; Fig. 4.6, column 1). Under these conditions, virus infectivity was inhibited >98%. Most of this inhibition could be explained by XPE's ability to block virus adsorption to host cells (Fig. 4.6, columns 1, 3, 4, 7), Interestingly, when XPE was only added to host cells prior to

virus inoculation and then removed, (Fig. 4.6, column 5) or after virus adsorption was complete (Fig. 4.6, column 6), virus infectivity could still be reduced by 20%, and 60%, respectively.

4.5 The inhibitory component in XPE is a lipid

In an effort to identify the active component of XPE responsible for its rather profound virus inhibitory activity, we subjected XPE to biochemical fractionation. XPE is composed of protein system and fat system; however, XHE has the same protein system but no fat system (Table. 4.3). Comparison of the inhibitory activity between XPE and XHE is shown in Fig. 4.7. Relative to XPE, XHE had detectable virus inhibitory activity at relatively high concentrations (250 mg/ml), but not at lower concentrations ($\leq$ 62.5 mg/ml). Based on this result, we hypothesize the fat system may contribute to the antiviral effect of XPE. To test this hypothesis, organic solvent of extraction of XPE was performed as described in the Material and Methods and both the organic and aqueous extracts were tested for antiviral activity (Fig. 4.8).

Compared to the aqueous extract, the organic extract of XPE (Fig. 4.8) exhibited an extraordinary antiviral activity, which can still inhibit approximately 90% of virus infection at a concentration of 490μg/ml. Aqueous extract of XPE have almost no inhibitory effect except at high concentrations. This result supports our pervious hypothesis that the fat system of XPE contributes to the antiviral activity of XPE.

4.6 Purification of antiviral lipid from XPE organic extracts

Individual lipids in XPE organic extracts were separated by analytical TLC and band visualized by primulin staining. Three distinct, primulin-staining bands (Mid1, Mid2, and sample origin), and a diffuse band containing multiple weakly stain bands (Top) were observed and labeled based on their relative chromatographic mobility (Fig. 4.9). A no-band zone (Bot), located between the sample origin and Mid1 bands also was identified so that all areas of the plate were represented during subsequent semi-preparative TLC, and analysis for antiviral activity. Semi-preparative TLC, band harvesting and testing for antiviral activity were performed as described in Materials and Methods. Following semi-preparative TLC, aliquots of each of the harvested areas (defined above) of the plate were re-chromatographed on analytical TLC plates along with the non-fractionated XPE organic extract so that degree of purity of each of the identified XPE lipid bands could be verified (Fig. 4.9). Note the sample origin band was not visible following semi-preparative TLC and re-chromatography on analytical plates. This result suggests the original primulin staining band seen following initial analytical TLC was likely due to sample residue at the origin rather than a unique non-mobile lipid. The antiviral activity of each of the bands/areas recovered from the semi-preparative TLC plate is shown in Figure 4.10. Only fraction Mid2 displayed antiviral activity. This fraction contained a single lipid band (Fig. 4.9) and was designated as lipid M2.

To further characterize the biological activity of lipid M2 and to determine whether it is responsible for the antiviral activity of XPE, we assessed whether its site of action was on H1N1 or host cells or both, as was the case for XPE using the indirect virus infectivity inhibition assay (Fig. 4.11). The antiviral activity of lipid M2 appeared identical to XPE including its ability to block virus infectivity following virus absorption and entry into the host cell. When lipid M2 is present in the cell culture medium, no matter if added before, during, or after virus adsorption, H1N1 infectivity was inhibited. As seen with XPE, the strongest inhibition was observed when lipid M2 was present at all three stages of the infectious cycle (Fig. 4.6, column 1). Thus, we conclude that lipid M2 fraction is primarily responsible for the antiviral activity of XPE.

4.7 Initial characterization of the lipid M2

In an effort to identity the chemical nature of lipid M2, we compared its chromatographic mobility on TLC to known commercial fatty acids (Fig. 4.12). Interestingly, while Lipid M1 appears to be oleic acid (optimal separation of each of the fatty acid standards was no achieved in this solvent system), lipid M2 did not co-chromatograph with any of the commercial lipids used as standards. It appears to have more polar character than any of the commercial fatty acid standards. To further characterize lipid M2, we compared the TLC profile of organic solvent extracts of XPE and XGE infant formulas (Fig. 4.13). Formulas XPE and XGE are composed of the same protein system, but different fat systems (Table 4.3). Compared to XPE, XGE lacks the predigested fat component. Considering the IC_{50} of XGE is 8-fold

lower than XPE (Table 4.2), the predigested fat maybe contributing the lipid component responsible the antiviral activity of XPE (i.e., lipid M2). As expected, XGE displayed no lipid bands on TLC plate while lipid M2 is clearly detected in XPE. These combined results indicate the inhibitory lipid M2 is a part of the predigested fat component of XPE and is thus likely a lipolysis-generated fatty acid. Characterization of purified lipid M2 by TLC using multiple solvent systems and additional fatty acid standards as well as by ESI Mass Spectrometry is ongoing.

4.8 Identification of inhibitory components in Human milk

Based on our results demonstrating the presence of antiviral lipids in some infant formula, we sought to determine if human milk also contained bioactive lipids that were at least partially responsible for its ability to inhibit H1N1 infectivity. Lipid extraction of human milk was performed as described in the Material and Methods and both the organic and aqueous extracts were tested for antiviral activity (Fig. 4.14). The organic solvent extract of human milk displayed potent, dose-dependent antiviral activity, as did whole human milk. The aqueous extract, however, contained little, if any virus inhibitory activity. Thus, lipids in human milk appear responsible for at least a major portion of its antiviral activity. These results, combined with the identification of the virus inhibitory lipid M2, strongly support the conclusion that certain lipids have antiviral activity including those contained in human milk and justify the need for further research aimed at understanding their mechanism of action and exploring their use as antimicrobial nutriceuticals.

4.9 Mechanism of H1N1 inhibition by lipid M2

The results above (Figs. 4.6, 4.11) suggest XPE (lipid M2) inhibits virus infectivity by acting directly on the virus and perhaps on MDCK cells as well. Since relatively little (20%) inhibition of virus infectivity is seen when MDCK cells are exposed to lipid M2 (Fig. 4.11) or XPE (Fig. 4.6) prior to virus inoculation, it is likely that most of the virus inhibitory activity is due to a direct action of lipid M2 on H1N1 resulting in blocking virus binding to host cells. The 20% inhibition seen during preincubation of MDCK cells with XPE or lipid M2 also could be due to direct interaction of the virus with residual lipid remaining bound to the host cell. To further address whether the inhibitory activity of Lipid M2is solely due to inhibition of virus binding or whether it can also inhibit the subsequent stages of virus entry or replication, we used modifications of the indirect virus infectivity inhibition assay (see Materials and Methods). For these experiments, virus was first incubated with MDCK monolayers at 4 C for 30 min to allow virus attachment and then Lipid M2 was added during virus entry as the cells were warmed to 35 C or during the virus replication stage (24 h incubation, 35 C) as described in Fig. 4.15.

When Lipid M2 is added during virus binding at 4 C, it blocks approximately 50% of subsequent virus infectivity (Fig. 4.15, column 1). If Lipid M2 is added following virus binding at 4 C and then the cells warmed to 35 C to allow virus entry, subsequent virus infectivity is inhibited by ~40% (Fig. 4.15, column 2), while the same Lipid M2 concentration (4 mg/ml) blocks>90% virus infectivity when added

during virus adsorption/entry at 35 C (Fig. 4.6, column 7). When Lipid M2 is added only during the final 24 h incubation at 35 C (virus replication stage) infectivity was inhibited by approximately 60% (Fig. 4.15, column 3, Fig. 4.6, column 6), the combined effects of Lipid M2 on both virus binding and entry (Fig. 4.15 columns 1 and 2) result in essentially 100% inhibition of infectivity and agrees with the result obtained when XPE (Lipid M2) is present through all stages of the virus infectivity cycle (Fig. 4.6, column 1). These results suggest Lipid M2 may inhibit virus infectivity not only by acting directly on the virus but also by at least partially inhibiting virus entry and perhaps subsequent virus replication. The proposed effect on virus replication; however, also may be at least partially explained by re-exposure of the newly formed virions to XPE or Lipid M2 as they emerge from infected cells.

4.10 Statistical analysis of the anti-viral effect of inhibitors

Statistical analysis of the effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. One-way ANOVA was used to analyze the effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. Viral infectivity means and standard deviations and 95% confidence interval for mean are shown in Table 4.4. The F values and significance levels resulting from these data appear in Table 4.5. The effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity is significantly different ($F(7, 30)=71.61$, $P < 0.001$, Table 4.5.).

A more detailed analysis, using Bonferroni's post hoc test, was conducted to test for differences between each treatment. Result of Bonferroni's post hoc test is presented in Table 4.6. Compared to negative control (NNN), all treatment conditions

significantly inhibited H1N1 infectivity ($P < 0.001$, respectively, Table 4.6.), except for XPE added before virus adsorption (YNN, $P=0.449$, Table 4.6.) and XPE added after virus adsorption (NNY, $P=1.000$, Table 4.6.). Among these treatment conditions that can significantly inhibit H1N1 infectivity, there is no significant difference.

Statistical analysis of the effect of various concentrations of XPE and XHE on H1N1 infectivity. The effect of various concentrations of XPE and XHE on H1N1 infectivity was analyzed by univariate analysis of variance. Descriptive statistics are shown in Table 4.7. Statistical analysis of the effect of concentration on the antiviral effect of XPE and XHE is shown in Table 4.8. The antiviral activity caused by XPE and XHE are significantly different ($F=494.568$, $P < 0.001$, Table 4.8.). Concentration also significantly influences the anti-viral effect of XPE and XHE ($F=48.653$, $P < 0.001$, Table 4.8.). Moreover, the extent of anti-viral effect of each inhibitor influenced by concentration is significantly different ($F=25.057$, $P < 0.001$, Table 4.8.). Compared to XHE, the antiviral effect of XPE is more significantly influenced by its concentration.

Statistical analysis of the effect of purified lipid fractions of XPE on H1N1 infectivity. The effect of purified lipid fractions of XPE on H1N1 infectivity was analyzed using a one-way ANOVA followed by a one-way ANOVA to determine differences on individual fractions. Viral infectivity means, standard deviations and 95% confidence interval for mean are shown in Table 4.9. The F values and significance

levels resulting from these data are shown in Table 4.10. The effect of purified lipid fractions of XPE on H1N1 infectivity is significantly different ($F(4, 5)=301.911$, $P < 0.001$, Table 4.10.). In order to compare the anti-viral effect caused by each purified lipid fraction, the Bonferroni's post hoc test was performed. Result of Bonferroni's post hoc test is shown in Table 4.11. The anti-viral effect of Mid2 lipid fraction is significantly higher than any other lipid fractions ($P < 0.001$, Table 4.11.). Additionally, there is no significant difference among Top, Mid1, Bot and Loading fractions.

Statistical analysis of the effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. To check for the effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity, a one-way ANOVA was performed. The effect of each treatment condition was then compared to each other using Bonferroni's post hoc test. Viral infectivity means and standard deviations and 95% confidence interval for mean are shown in Table 4.12.The F values and significance levels resulting from these data are shown in Table 4.13.There are significant differences in the anti-viral effect caused by lipid M2 different treatment conditions ($F(7,32)=162.227$, $P < 0.001$, Table 4.13.). Using Bonferroni's post hoc test, the difference between each treatment condition with lipid M2 was analyzed. Compared to negative control (NNN), all the lipid M2 treatment conditions have significant anti-viral effect ($P <0.05$, Table 4.14.), except for the condition when lipid M2 is added only after virus adsorption (NNY, $P=0.323$, Table 4.14.). Among these effective conditions, the greatest anti-viral effect

was observed when lipid M2 present during the virus adsorption, no matter if added before, and after virus adsorption (YYY, YYN, NYY, NYN, $P < 0.001$, respectively Table 4.14.). And there is no significant difference between these four treatment conditions ($P = 1$, respectively, Table 4.14.)

Statistical analysis of the effect of Lipid M2 on H1N1 Binding to MDCK cells. The Effect of Lipid M2 on H1N1 Binding to MDCK cells was analyzed by one-way ANOVA. Descriptive statistics are shown in Table 4.15. The F values and significance levels are shown in Table 4.16. The effect of Lipid M2 on H1N1 Binding to MDCK cells is significantly different between treatment conditions ($F(4, 19)=72.823$, $P < 0.001$, Table 4.16.). Bonferroni's post hoc test was conducted to test for differences between anti-viral effects in each treatment conditions. Compared to negative control (NNN), all the treatment conditions have significant antiviral effect ($P < 0.001$, respectively, Table 4.17.). Among these treatment conditions, lipid M2 added during viral entry and replication (NYY) has the highest antiviral effect ($P < 0.005$, Table 4.17).

4.11 Figures and Tables

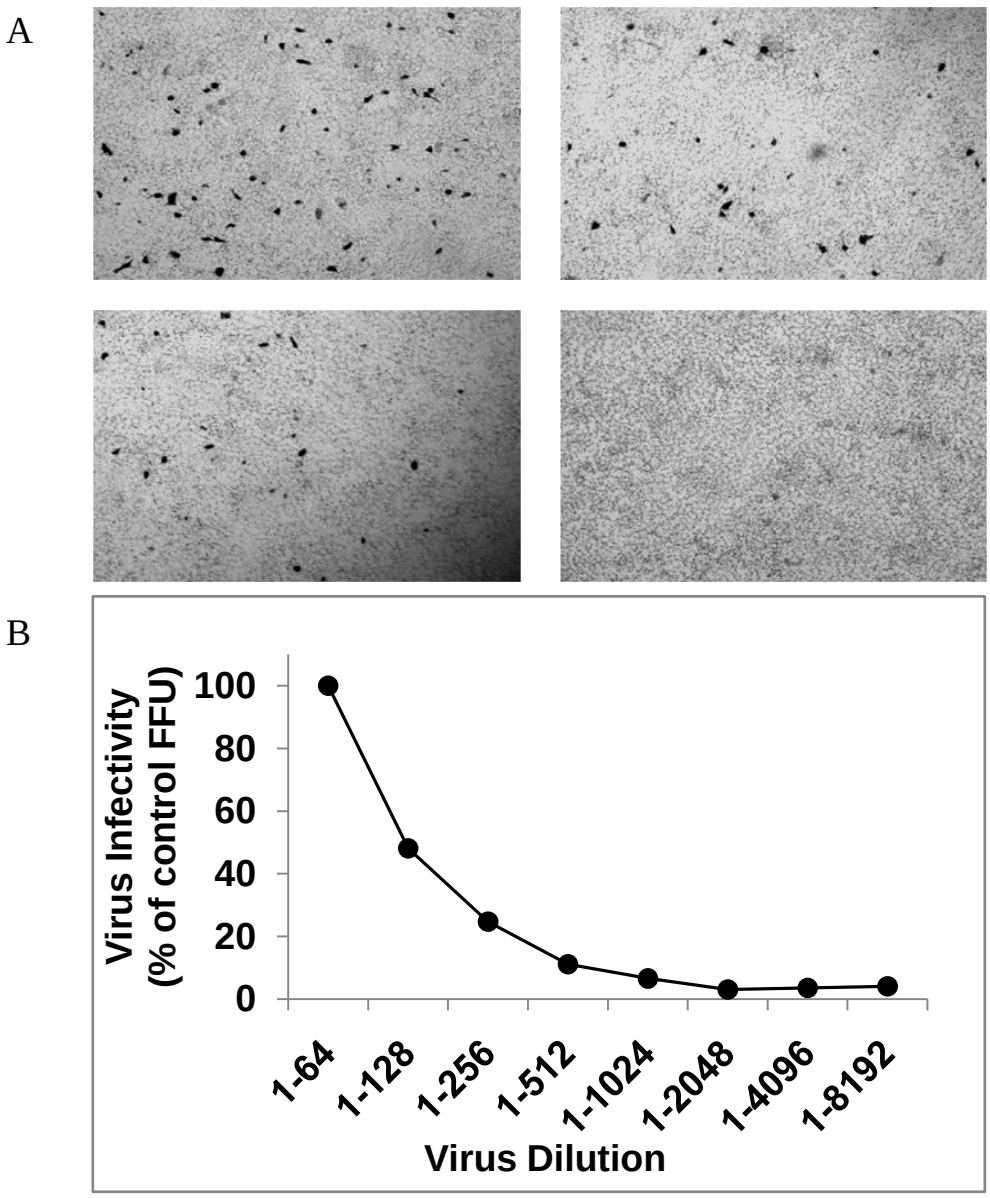


Figure 4.1. H1N1 infectivity of MDCK cells is proportional to virus concentration. Inoculation of MDCK monolayers with H1N1 virus, detection, and measurement of infected cell foci was performed as described in Materials and Methods. (A), phase contrast photomicrographs show distinct foci of infection that increase in number upon increasing concentration of inoculating virus (left to right, 4 fold virus dilution series, with the lower right image representing the negative control (no virus)). (B), graph demonstrates linearity of observed FFU with relative virus concentration. The relative virus concentration varied from 16-397 FFU units.

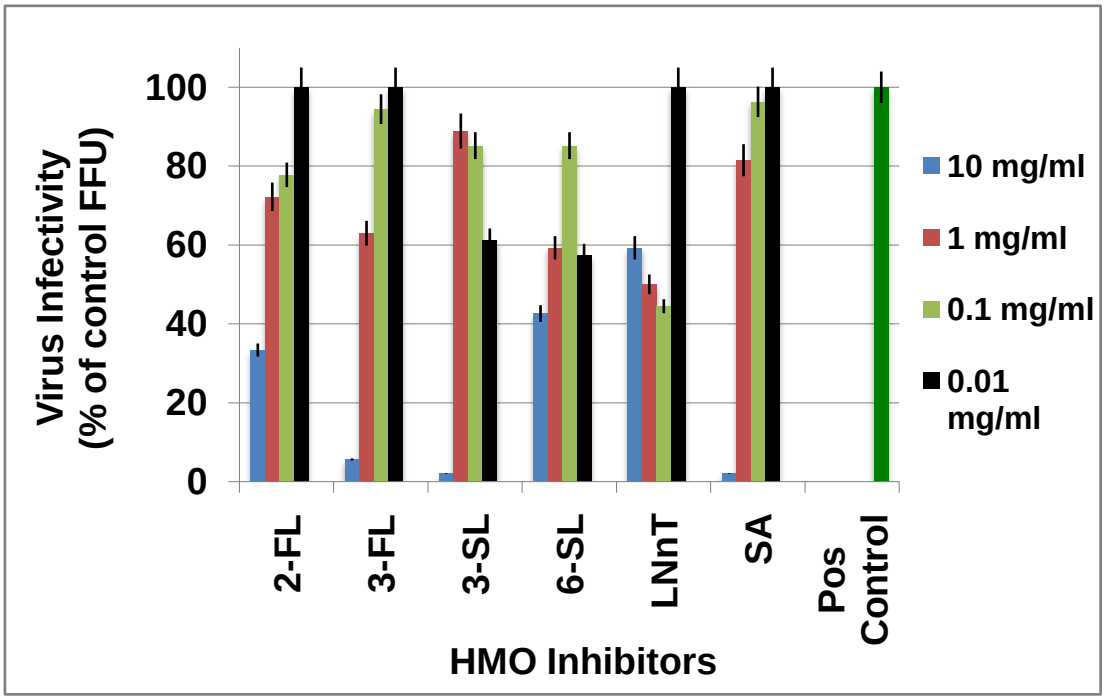


Figure 4.2. Effect of various HMO on H1N1 virus infectivity. Virus infectivity was measured in the presence and absence of the indicated oligosaccharides as described in Materials and Methods. Each oligosaccharide was tested in duplicate at four concentrations, 0.01, 0.1, 1.0, and 10 mg/ml.

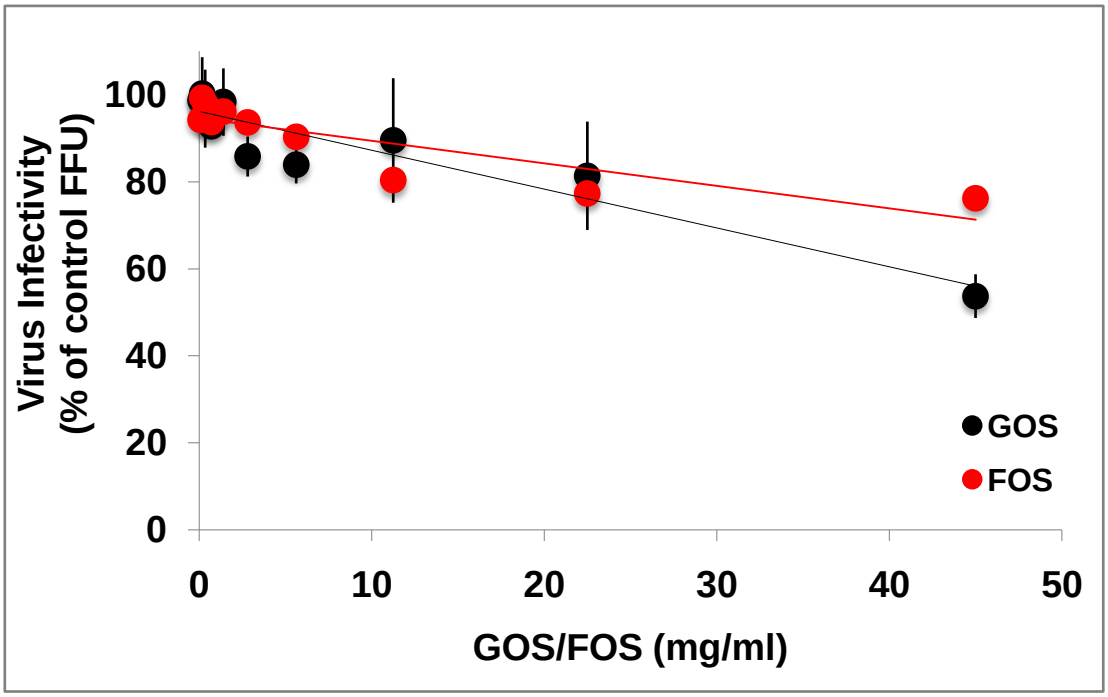


Figure 4.3. Effect of prebiotics Gos and Fos on H1N1 infectivity. Virus infectivity was measured in the presence and absence of the indicated concentrations of GOS and FOS prebiotic oligosaccharides as described in Materials and Methods. (•), FOS; (•), GOS.

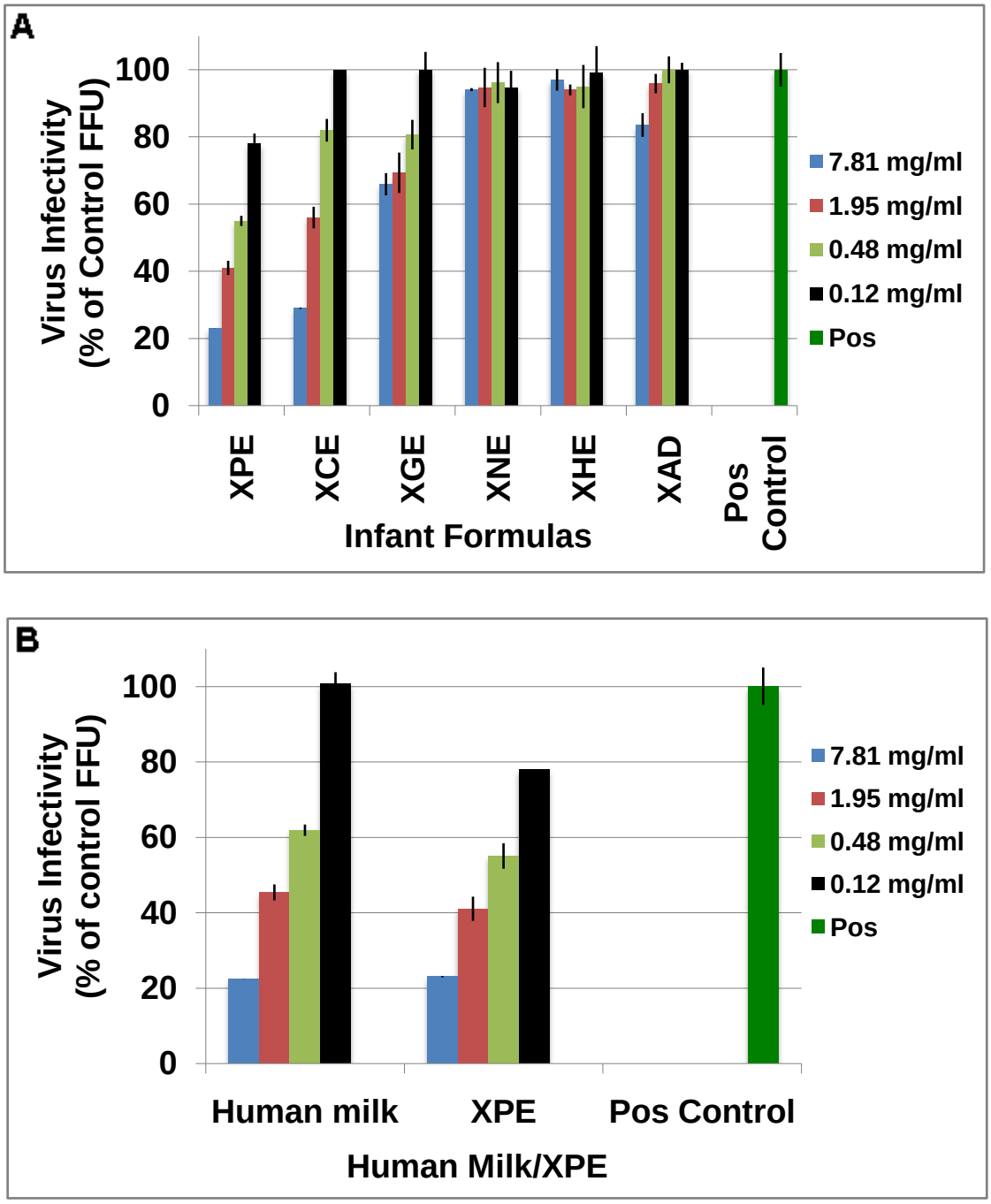


Figure 4.4. Effect of various infant formulas and human milk on H1N1 virus infectivity. Virus infectivity was measured in the presence and absence of the indicated concentrations of various infant formula or human milk as described in Materials and Methods. Each compound was tested in duplicate at the indicated concentrations. (A), comparison of virus inhibitory activity of various proprietary infant formulas; (B), comparison of the virus inhibitory activity of XPE (the most inhibitory formula) and human milk.

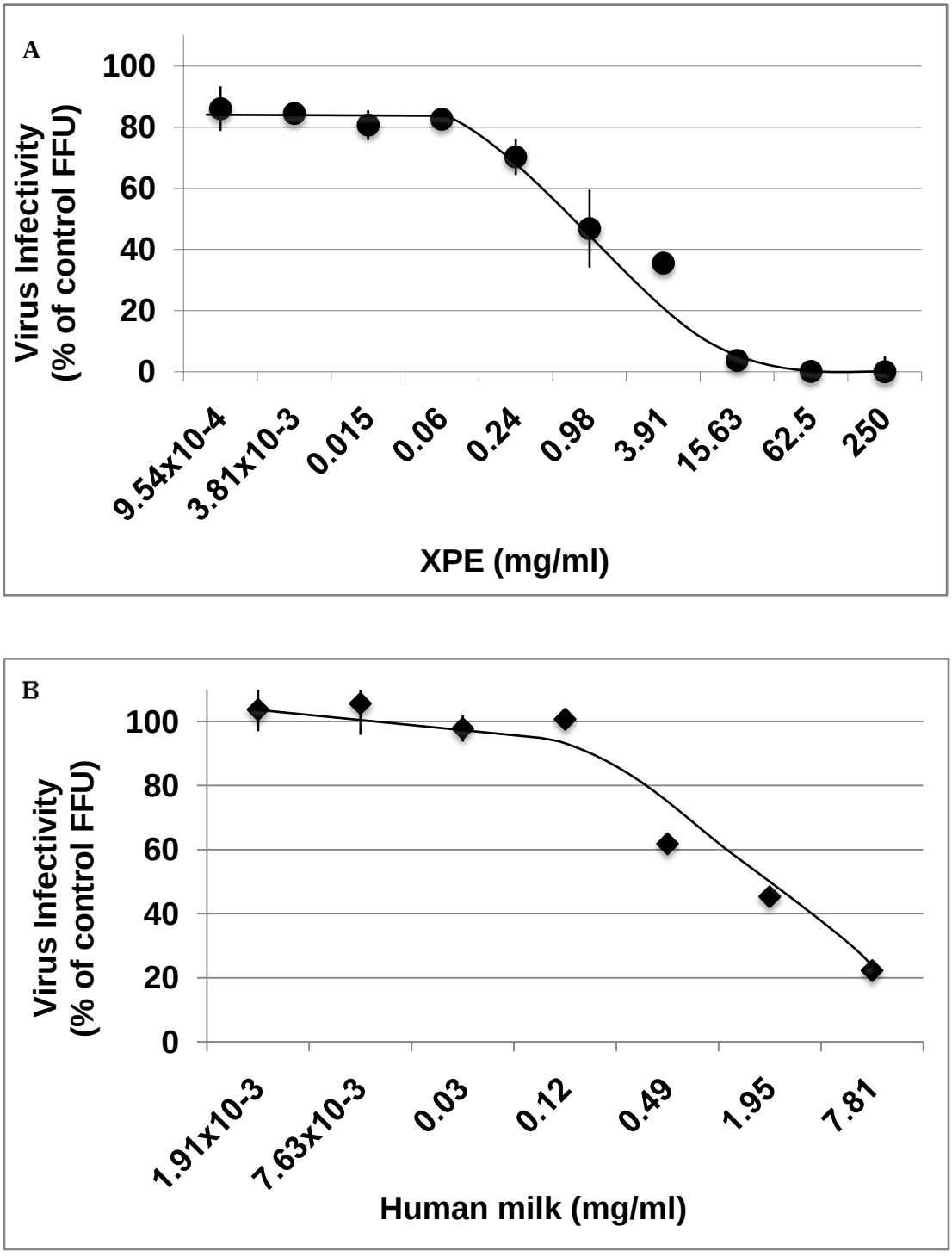
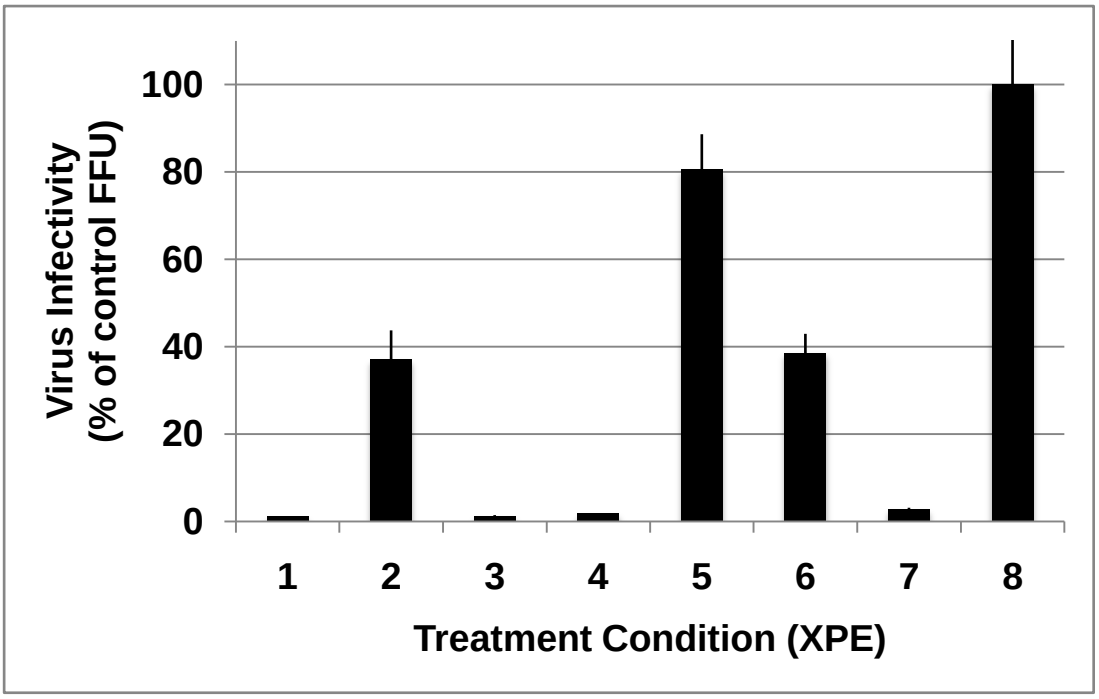


Figure 4.5. Comparison of the effects of various concentrations of XPE and human milk on H1N1 infectivity. Virus infectivity was measured in the presence and absence of the indicated concentrations of XPE and human milk as described in Materials and Methods. Each of the concentrations of XPE and milk were tested in duplicate.



Column No	1	2	3	4	5	6	7	8
Pretreat 5 h	Y	Y	N	Y	Y	N	N	N
XPE in adsorption	Y	N	Y	Y	N	N	Y	N
XPE in medium 24h	Y	Y	Y	N	N	Y	N	N

Figure 4.6. Effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. The effect of different XPE treatment conditions (as denoted in the table above) on virus infectivity of MDCK cells were measured using the indirect virus infectivity inhibitory assay as described in Materials and Methods. Pretreat 5h: MDCK cells were preincubated 5 h with XPE in MEM-serum, at 35 C; XPE in adsorption: XPE was added to H1N1, incubated for 30 min at 35 C and then added to MDCK cells as described in Materials and Methods; XPE in medium 24h: XPE was added after virus adsorption to MDCK cells during the final 24 h virus replication incubation as described in Materials and Methods. XPE was used at a concentration of 15.64 mg/ml.

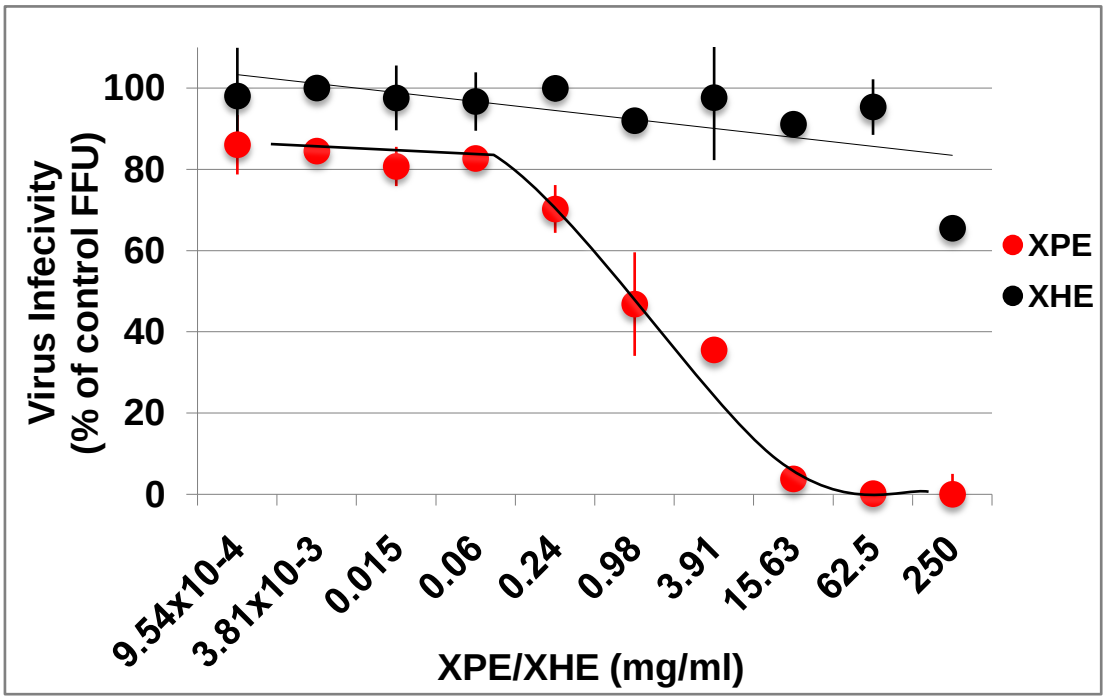


Figure 4.7. Effect of various concentrations of XPE and XHE on H1N1 infectivity. Virus infectivity was measured in the presence and absence of the indicated concentrations of XPE and XHE using the direct virus infectivity inhibition assay as described in Materials and Methods. Each compound concentration was tested in duplicate.

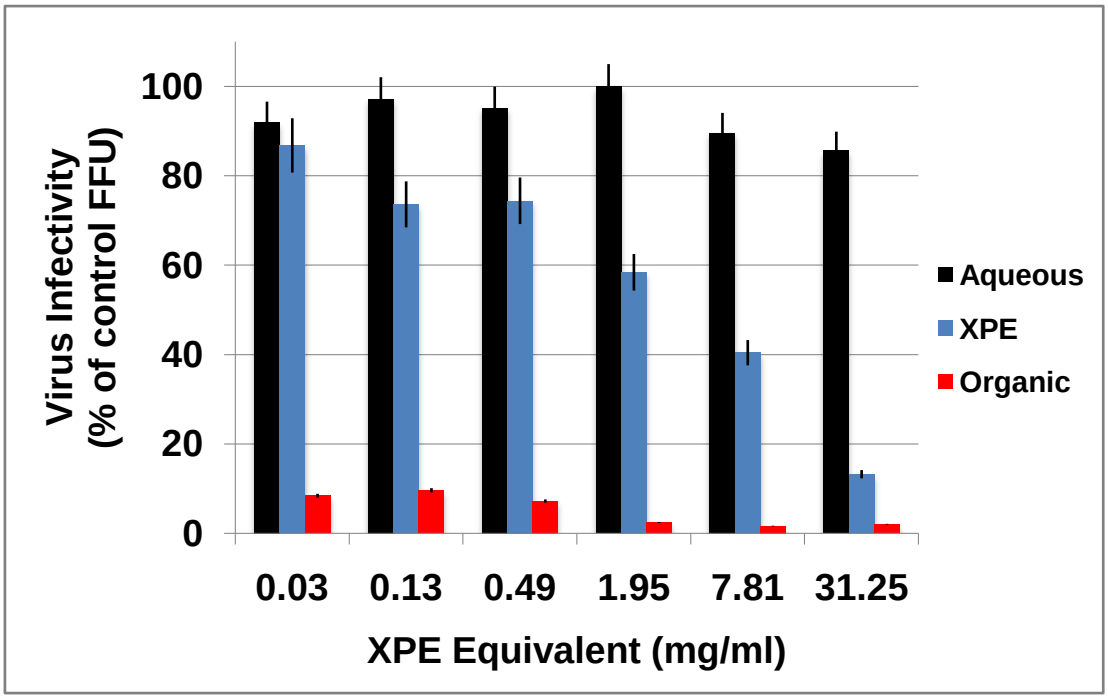


Figure 4.8. Effect of XPE fractions on H1N1 infectivity. Virus infectivity was measured using the direct virus infectivity inhibition assay in the presence and absence of the indicated concentrations of intact XPE and its aqueous or organic extract fractions. Organic solvent extraction of XPE (0.75 g/0.75 ml) and measurement of virus infectivity was done as described in Materials and Methods. The XPE organic and aqueous extracts were diluted to equal the original intact XPE concentration (XPE Equivalent) so as to directly compare the relative inhibitory activity of each fraction. Five replicates of each compound were tested.

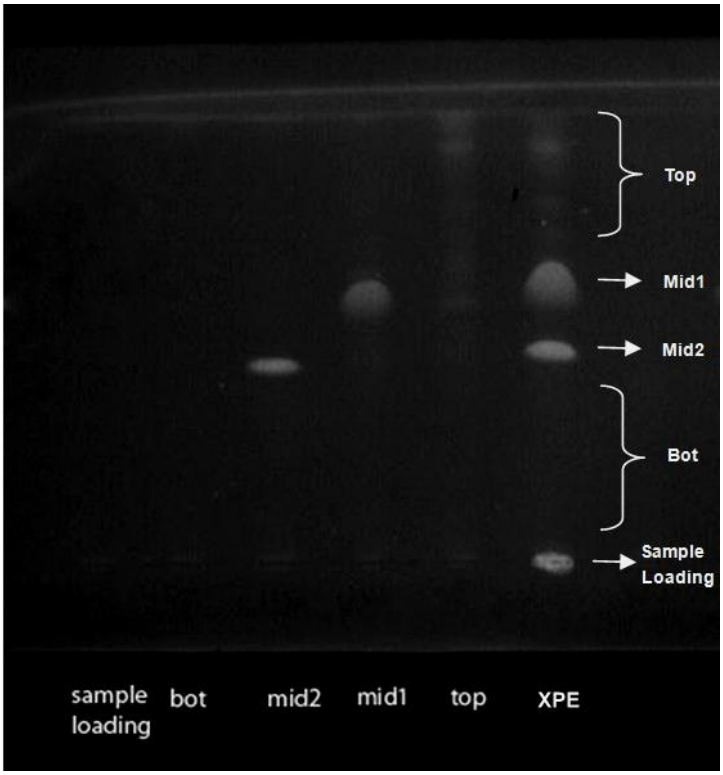


Figure 4.9. Purification of lipid fractions from XPE organic solvent extract. The organic extract of XPE was prepared and subjected to semi-preparative TLC as described in Materials and Methods. All lipid staining and blank areas were scraped from the semi-preparative TLC plate and aliquots re-chromatographed on analytical TLC plates (shown in the above chromatogram). Note: each area recovered from the semi-preparative TLC plate (as denoted on the right edge of the figure) contained purified lipid band that displayed chromatographic mobility identical to those seen in the original XPE organic extract. XPE: organic extract of XPE; top, mid1, mid2, bot, and sample loading lances correspond to the areas scraped from the semi-preparative TLC plate and re-chromatographed on the analytical TLC plate shown in this figure.

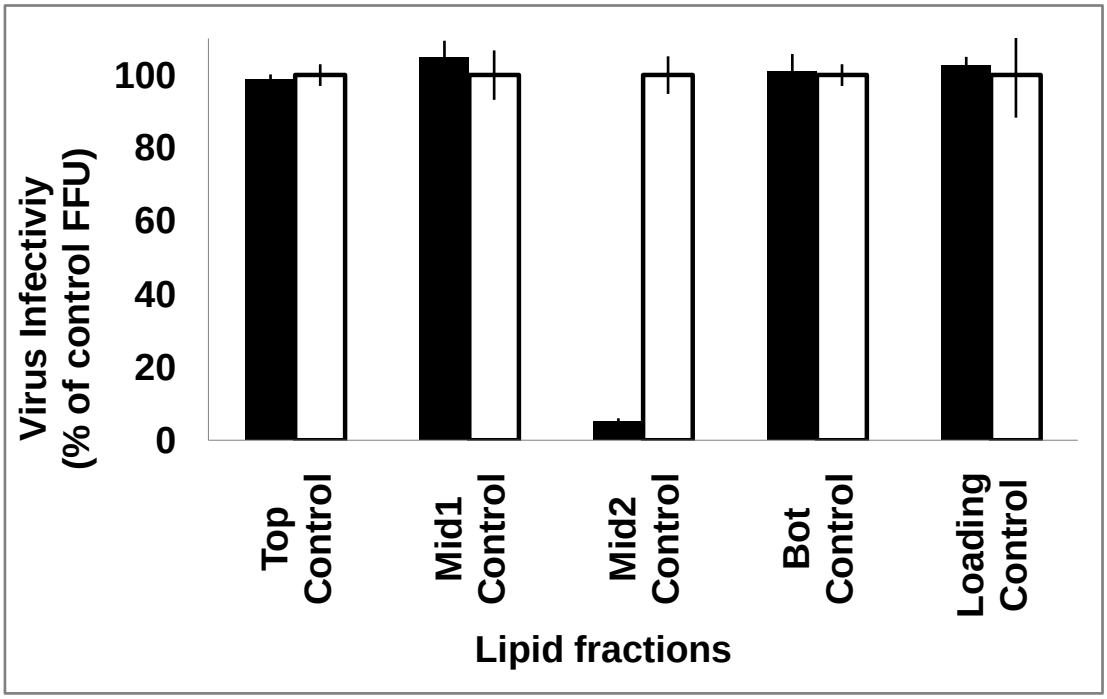
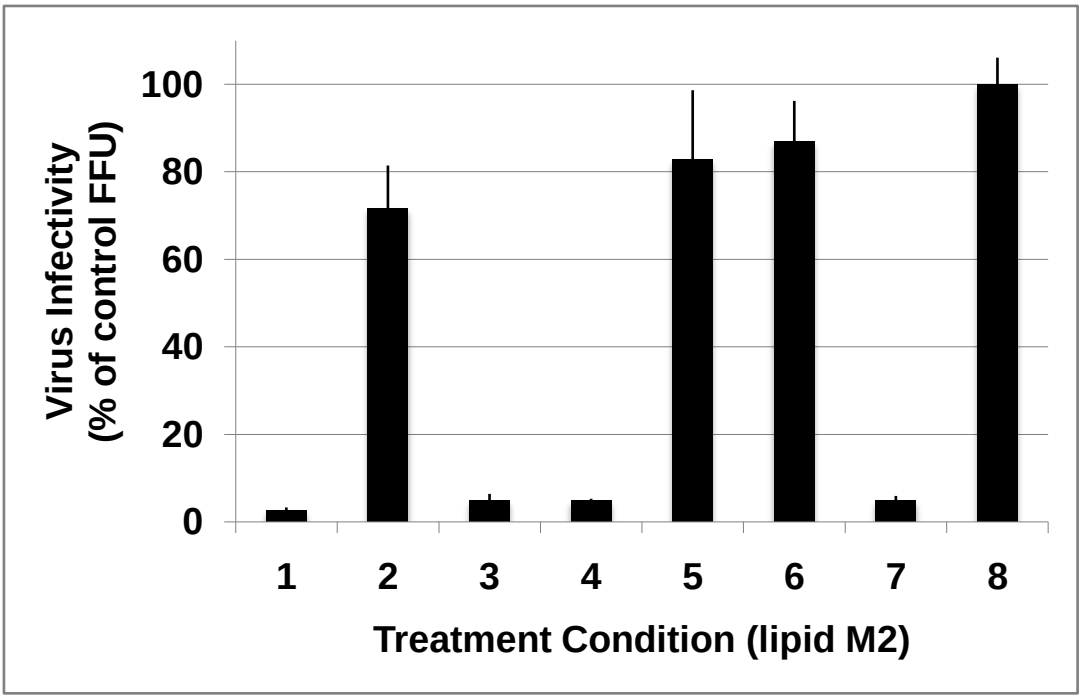


Figure 4.10. Effect of purified lipid fractions of XPE on H1N1 infectivity. Virus infectivity was measured in the presence and absence of purified lipid fractions of XPE (as shown in Figure 4.9. above) as described in Materials and Methods. Each compound was tested in five-replicates at the XPE equivalent concentration of 4 mg/ml.



Column No	1	2	3	4	5	6	7	8
Pretreat 5 h	Y	Y	N	Y	Y	N	N	N
Lipid M2 in adsorption	Y	N	Y	Y	N	N	Y	N
Lipid M2 in medium 24h	Y	Y	Y	N	N	Y	N	N

Figure 4.11. Effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. The effect of different lipid M2 treatment conditions (as denoted in the table above) on virus infectivity of MDCK cells were measured using the indirect virus infectivity inhibitory assay as described in Materials and Methods. Pretreat 5h: MDCK cells were preincubated 5 h with lipid M2 in MEM-serum, at 35 C; Lipid M2 in adsorption: Lipid M2 was added to H1N1, incubated for 30 min at 35 C and then added to MDCK cells as described in Materials and Methods; Lipid M2 in medium 24h: Lipid M2 was added after virus adsorption to MDCK cells during the final 24 h virus replication incubation as described in Materials and Methods. Lipid M2 was used at a concentration of 4 mg/ml XPE equivalent.

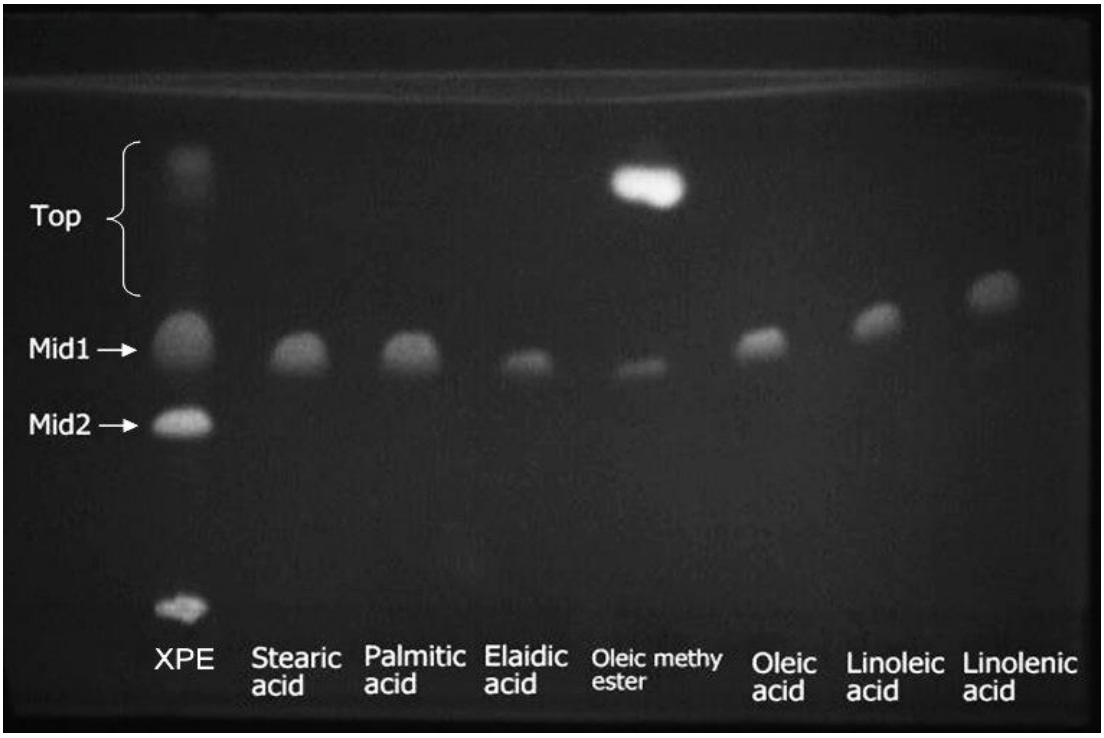


Figure 4.12. Comparison of the chromatographic mobility of Lipid M2 and known fatty acids. Commercial fatty acids (30-50 nmol) were applied to analytical TLC plates as described in Materials and Methods. Bands were detected by primulin spray.

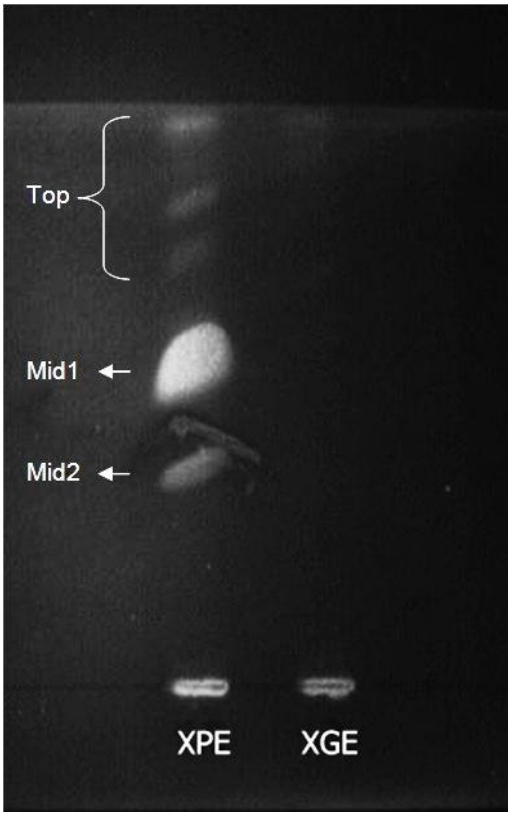


Figure 4.13. Comparison of lipid profiles of XPE and XGE. XPE and XGE were extracted and aliquots of the organic solvent phase analyzed by analytical TLC as described in the Materials and Methods. Bands were visualized by primulin spray.

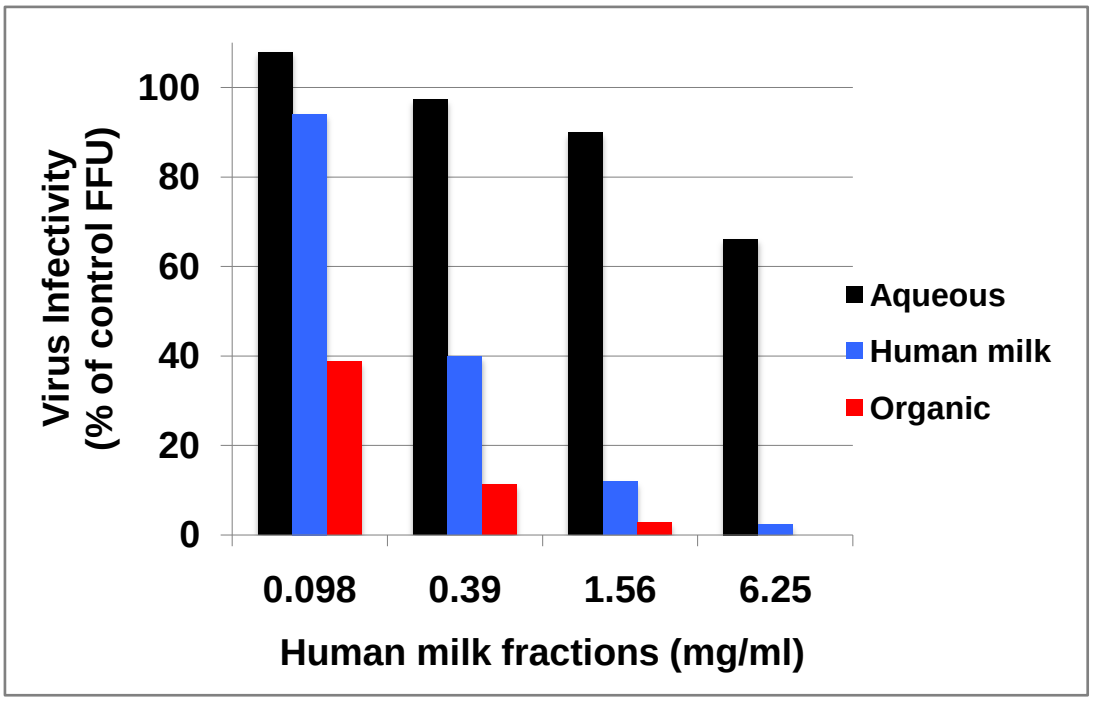
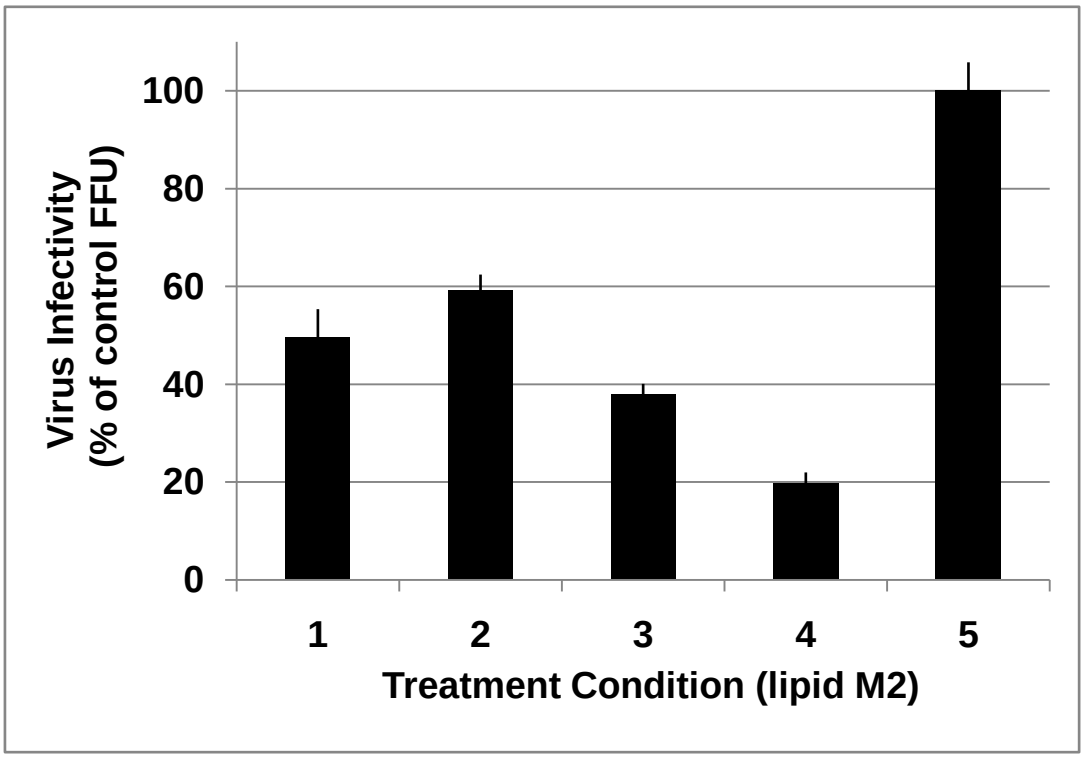


Figure 4.14. Effect of human milk fractions on HPIV3 infectivity. Human milk was subjected to organic solvent extraction and the effect of various concentrations of the aqueous, organic, and whole (un-extracted) milk fractions on H1N1 virus infectivity was measured using the direct virus infectivity inhibition assay as described in Materials and Methods. (■), aqueous phase; (■), organic phase, (■), intact milk.



Column No	1	2	3	4	5
Virus binding at 4 C 30min	**Y**	**N**	**N**	**N**	**N**
Virus entry at 35 C 30min	**N**	**Y**	**N**	**Y**	**N**
Virus replication 24h	**N**	**N**	**Y**	**Y**	**N**

Figure 4.15. Effect of Lipid M2 on H1N1 Binding to MDCK cells. The effect of different lipid M2 treatment conditions (as denoted in the table above) on virus infectivity of MDCK cells was measured using the indirect virus infectivity inhibitory assay as described in Materials and Methods, except this virus binding was performed at 4 C rather than 35 C. Virus binding at 4 C: H1N1 virus was added to MDCK cells and incubated at 4 C for 30 min; Virus entry at 35 C 30min: MDCK monolayers were washed twice with cold PBS and then XPE was added to MDCK cells, incubated at 35 C for 30 min; Virus replication 24h: Lipid M2 was added after virus adsorption to MDCK cells during the final 24 h virus replication incubation.

Concentration for 50% Inhibition of Infectivity	
Inhibitors	**Concentration**
GOS	~ 50 mg/ml*
FOS	$^{+}$NI
LNY	> 10 mg/ml
6-SL	10 mg/ml
2-FL	~ 7.5 mg/ml
3-SL	~ 5 mg/ml
3-FL	~ 5 mg/ml
SA	~ 7.5 mg/ml
* wet weight, $^{+}$NI= no inhibition	

Table 4.1. Summary of effect of selected oligosaccharides on *in vitro* H1N1 infectivity. The relative inhibitory effects of various HMO and prebiotic oligosaccharides on H1N1 infectivity were evaluated by comparison of their IC_{50} values. Each oligosaccharide was tested in duplicate at various concentrations ranging from 0.01 to 10 mg/ml as described in Materials and Methods.

Concentration for 50% Inhibition of Infectivity	
Inhibitor	**Concentration**
XAD	15.63 mg/ml
XPE	0.98 mg/ml
XGE	7.81 mg/ml
XHE	+NI
XCE	4.59 mg/ml
Human Milk	1.95 mg/ml
* wet weight, $^{+}$NI= no inhibition	

Table 4.2. Summary of effect of various infant formulas and human milk on H1N1 infectivity. The relative inhibitory effects of infant formulas and human milk on H1N1 infectivity were evaluated by comparison of their IC_{50} values. Each inhibitor was tested in duplicate at various concentrations to determine IC_{50} values as described in Materials and Methods.

Sample	Protein composition (per 100ml)	Fat composition (per 100ml)
XPE	1.5g hydrolyzed whey protein	2.17g triglycerides +1.84g predigested fat
XHE	1.5g hydrolyzed whey protein	None
XGE	1.5g hydrolyzed whey protein	3.6 g hydrolyzed whey protein No monoglycerides or lecithin

Table 4.3. Comparison of the protein and lipid composition of XPE, XHE and XGE. Note: XHE contains only a protein component.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
YYY	5	1.18	0.0643	0.0287	1.1	1.26	1.12	1.25
YNY	4	36.5	6.69	3.34	26.8	40.1	27.5	42
NYY	4	1.2	0.075	0.0375	1.08	1.32	1.12	1.3
YYN	5	1.81	0.0986	0.0441	1.69	1.93	1.74	1.98
YNN	5	80.6	18	8.07	58.2	103	65.6	112
NNY	5	38.4	9.05	4.52	28.3	46.7	26.7	46.8
NYN	5	2.81	0.324	0.145	2.4	3.21	2.42	3.23
NNN	5	100	19.1	8.52	76.3	124	82	125
Total	38	40.8	45.6	7.39	25.8	55.8	1.12	125

Table 4.4. Descriptive Statistics for the effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. FFU means, standard deviation, and 95% confidence intervals for means were calculated by SPSS. N: sample size; Std.: standard deviation.

	Sum of Squares	df	Mean Square	F	P
Between Groups	72500	7	10400	71.6	0.000
Within Groups	4340	30	145		
Total	76800	37			

Table 4.5. One-way ANOVA test for the effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. F and P (Sig.) values were conducted through one-way ANOVA. Sig.: significance level; df: degrees of freedom.

A

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
YYY	**YNY**	-27.3	8.07	0.056	-55	0.35
	NYY	-0.0215	8.07	1	-27.7	27.6
	YYN	-0.636	7.61	1	-26.7	25.4
	YNN	-79.4	7.61	0	-105	-53.3
	NNY	-38.8	7.61	0	-24.9	-46.7
	NYN	-1.63	7.61	1	-27.7	24.4
	NNN	-98.8	7.61	0	-125	-72.7
YNY	**YYY**	27.3	8.07	0.056	-0.35	55
	NYY	27.3	8.5	0.089	-1.87	56.4
	YYN	26.7	8.07	0.069	-0.986	54.3
	YNN	-52.1	8.07	0	-79.8	-24.4
	NNY	-71.5	8.07	0	-99.2	-43.9
	NYN	25.7	8.07	0.095	-1.98	53.3
	NNN	-71.5	8.07	0	-99.2	-43.9
NYY	**YYY**	0.0215	8.07	1	-27.6	27.7
	YNY	-27.3	8.5	0.089	-56.4	1.87
	YYN	-0.615	8.07	1	-28.3	27
	YNN	-79.4	8.07	0	-107	-51.7
	NNY	-98.8	8.07	0	-126	-71.1
	NYN	-1.61	8.07	1	-29.3	26.1
	NNN	-98.8	8.07	0	-126	-71.1
YYN	**YYY**	0.636	7.61	1	-25.4	26.7
	YNY	-26.7	8.07	0.069	-54.3	0.986
	NYY	0.615	8.07	1	-27	28.3
	YNN	-78.8	7.61	0	-105	-52.7
	NNY	-98.2	7.61	0	-124	-72.1
	NYN	-27.3	8.07	1	-55	0.35
	NNN	-0.0215	8.07	0	-27.7	27.6

Table 4.6 (cont. on next page)

B

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
YNN	**YYY**	79.4	7.61	0.000	53.3	105
	YNY	52.1	8.07	0.000	24.4	79.8
	NYY	79.4	8.07	0.000	51.7	107
	YYN	78.8	7.61	0.000	52.7	105
	NNY	-19.4	7.61	0.449	-45.5	6.66
	NYN	77.8	7.61	0.000	51.7	104
	NNN	-19.4	7.61	0.449	-45.5	6.66
NNY	**YYY**	98.8	7.61	0.000	72.7	125
	YNY	71.5	8.07	0.000	43.9	99.2
	NYY	98.8	8.07	0.000	71.1	126
	YYN	98.2	7.61	0.000	72.1	124
	YNN	19.4	7.61	0.449	-6.66	45.5
	NYN	97.2	7.61	0.000	71.1	123
	NNN	0	7.61	1.000	-26.1	26.1
NYN	**YYY**	1.63	7.61	1.000	-24.4	27.7
	YNY	-25.7	8.07	0.095	-53.3	1.98
	NYY	1.61	8.07	1.000	-26.1	29.3
	YYN	0.994	7.61	1.000	-25.1	27.1
	YNN	-77.8	7.61	0.000	-104	-51.7
	NNY	-97.2	7.61	0.000	-123	-71.1
	NNN	-97.2	7.61	0.000	-123	-71.1
NNN	**YYY**	98.8	7.61	0.000	72.7	125
	YNY	71.5	8.07	0.000	43.9	99.2
	NYY	98.8	8.07	0.000	71.1	126
	YYN	98.2	7.61	0.000	72.1	124
	YNN	19.4	7.61	0.449	-6.66	45.5
	NNY	0	7.61	1.000	-26.1	26.1
	NYN	97.2	7.61	0.000	71.1	123
*. The mean difference is significant at the 0.05 level.						

Table 4.6. Bonferroni's post hoc test for the effect of different XPE treatment conditions of MDCK Cells on H1N1 infectivity. Bonferroni's post hoc test was conducted to compare difference between each treatment condition. P (Sig).: significance level; df: degrees of freedom. For ease of reading, comparison of the eight different treatment groups are presented in two sets of four groups in tables, A and B.

A

Dependent Variable: Viral Infectivity				
Concentration	**Inhibitor**	**Mean**	**Std. Deviation**	**N**
250 mg/ml	XPE	0	0	2
	XHE	65.5	11.9	2
	Total	32.7	38.4	4
62.5 mg/ml	XPE	0.105	0.0495	2
	XHE	95.3	0	2
	Total	47.7	55	4
15.63 mg/ml	XPE	3.74	0.403	2
	XHE	91.1	7.98	2
	Total	47.4	50.7	4
3.91 mg/ml	XPE	35.5	1.99	2
	XHE	97.7	7.18	2
	Total	66.6	36.1	4
0.98 mg/ml	XPE	46.8	0.75	2
	XHE	91.9	3.19	2
	Total	69.4	26.1	4
0.24 mg/ml	XPE	70.2	12.7	2
	XHE	99.9	2.86	2
	Total	85.1	18.7	4

Table 4.7 (cont. on next page)

B

Dependent Variable: Virus Infectivity				
Concentration	**Inhibitor**	**Mean**	**Std. Deviation**	**N**
0.06 mg/ml	XPE	82.6	5.88	2
	XHE	96.7	15.4	2
	Total	89.7	12.5	4
0.015 mg/ml	XPE	80.7	0.75	2
	XHE	97.6	1.15	2
	Total	89.1	9.78	4
3.81x10-3 mg/ml	XPE	84.5	4.84	2
	XHE	100	6.84	2
	Total	92.3	10.2	4
9.54x10-4 mg/ml	XPE	86.1	1.24	2
	XHE	98.1	2.96	2
	Total	92.1	7.18	4
Total	XPE	49	36.1	20
	XHE	93.4	11.4	20
	Total	71.2	34.7	40

Table 4.7. Descriptive Statistics for the effect of various concentrations of XPE and XHE on H1N1 infectivity. FFU means and standard deviations were calculated by SPSS. N: sample size; Std.: standard deviation.

Dependent Variable: Inhibition					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P**
Corrected Model	46100	19	2420	60.9	0.000
Intercept	203000	1	203000	5100	0.000
Concentration	17400	9	1940	48.7	0.000
Inhibitor	19700	1	19700	495	0.000
Concentration * Inhibitor	8970	9	997	25.1	0.000
Error	795	20	39.8		
Total	250000	40			
Corrected Total	46800	39			
a. R Squared = .983 (Adjusted R Squared = .967)					

Table 4.8. Tests of Between-Subjects Effects for the effect of various concentrations of XPE and XHE on H1N1 infectivity. The effects of different inhibitors and various concentrations on H1N1 infectivity are analyzed. P (Sig).: significance level; df: degrees of freedom.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Top	2	257	3.54	2.5	225	288	254	259
Mid1	2	250	12	8.5	142	358	241	258
Mid2	2	11.5	2.12	1.5	7.56	30.6	10	13
Bot	2	234	12.7	9	120	348	225	243
Loading	2	241	6.36	4.5	183	298	236	245
Total	10	198	99	31.3	128	269	10	259

Table 4.9. Descriptive Statistics for the effect of purified lipid fractions of XPE on H1N1 infectivity. FFU means, standard deviation, and 95% confidence interval for mean were calculated by SPSS. N: sample size; Std.: standard deviation.

	Sum of Squares	df	Mean Square	F	P
Between Groups	87900	4	22000	302	87900
Within Groups	364	5	72.8		364
Total	88300	9			88300

Table 4.10. One-way ANOVA test for the effect of purified lipid fractions of XPE on H1N1 infectivity. F and P (Sig.) value were conducted through one-way ANOVA. Sig.: significance level; df: degrees of freedom.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
YNN	6	49.6	5.77	2.36	43.5	55.6	44.6	59.5
NYN	5	59.1	3.26	1.46	55.1	63.2	55	63.8
NNY	4	38	2.06	1.03	34.7	41.3	35	39.4
NYY	5	19.8	2.14	0.956	17.2	22.5	17.6	22.4
NNN	4	95	14.7	7.35	71.6	118	73.8	108
Total	24	51	25.1	5.13	40.4	61.6	17.6	108

Table 4.11. Descriptive Statistics for Effect of Lipid M2 on H1N1 Binding to MDCK cells. FFU means, standard deviations, and 95% confidence intervals for mean were calculated by SPSS. N: sample size; Std.: standard deviation.

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
Top	Mid1	7	8.53	1	-33.7	47.7
	Mid2	245	8.53	0!	204	286
	Bot	22.5	8.53	0.461	-18.2	63.2
	Loading	16	8.53	1	-24.7	56.7
Mid1	Top	-7	8.53	1	-47.7	33.7
	Mid2	238	8.53	0	197	279
	Bot	15.5	8.53	1	-25.2	56.2
	Loading	9	8.53	1	-31.7	49.7
Mid2	Top	-245	8.53	0	-286	-204
	Mid1	-238	8.53	0	-279	-197
	Bot	-223	8.53	0	-263	-182
	Loading	-229	8.53	0!	-270	-188
Bot	Top	-22.5	8.53	0.461	-63.2	18.2
	Mid1	-15.5	8.53	1	-56.2	25.2
	Mid2	223	8.53	0	182	263
	Loading	-6.5	8.53	1	-47.2	34.2
Loading	Top	-16	8.53	1	-56.7	24.7
	Mid1	-9	8.53	1	-49.7	31.7
	Mid2	229	8.53	0	188	270
	Bot	6.5	8.53	1	-34.2	47.2
*. The mean difference is significant at the 0.05 level.						

Table 4.12. Bonferroni's post hoc test for the effect of purified lipid fractions of XPE on H1N1 infectivity. Bonferroni's post hoc test was conducted to compare difference between each treatment condition. P (Sig).: significance level; df: degrees of freedom.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
YYY	5	19.2	5.4	2.4	12.5	25.9	12	24
YNY	5	530	73	32.8	441	620	459	630
NYY	5	36.2	11.2	5	22.3	50	27	55
YYN	5	36.4	2.97	1.33	32.7	40.1	32	40
YNN	5	620	117	53	469	760	424	720
NNY	5	650	69	31.1	560	730	540	720
NYN	5	36	8.1	3.63	25.9	46.1	27	47
NNN	5	740	45.5	20.4	690	800	660	770
Total	40	333	314	49.6	232	433	12	770

Table 4.13. Descriptive Statistics for effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. FFU means, standard deviations, and 95% confidence intervals for mean were calculated by SPSS. N: sample size; Std.: standard deviation.

	Sum of Squares	df	Mean Square	F	P
Between Groups	3730000	7	533000	162	
Within Groups	105000	32	3290		
Total	3840000	39			

Table 4.14. One-way ANOVA test for the effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. F and P (Sig.) value were conducted through one-way ANOVA. Sig.: significance level; df: degrees of freedom.

A

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
YYY	**YNY**	-512	36.3	0	-636	-389
	NYY	-17	36.3	1	-141	107
	YYN	-17.2	36.3	1	-141	106
	YNN	-596	36.3	0	-720	-472
	NNY	-626	36.3	0	-750	-502
	NYN	-16.8	36.3	1	-140	107
	NNN	-723	36.3	0	-847	-600
YNY	**YYY**	512	36.3	0	389	636
	NYY	495	36.3	0	372	619
	YYN	495	36.3	0	372	619
	YNN	-83.6	36.3	0.778	-207	40
	NNY	-114	36.3	0.103	-237	9.99
	NYN	496	36.3	0	372	619
	NNN	-211	36.3	0	-334	-87.2
NYY	**YYY**	17	36.3	1	-107	141
	YNY	-495	36.3	0	-619	-372
	YYN	-0.2	36.3	1	-124	123
	YNN	-579	36.3	0	-703	-455
	NNY	-609	36.3	0	-733	-485
	NYN	0.2	36.3	1	-123	124
	NNN	-706	36.3	0	-830	-583
YYN	**YYY**	17.2	36.3	1	-106	141
	YNY	-495	36.3	0	-619	-372
	NYY	0.2	36.3	1	-123	124
	YNN	-579	36.3	0	-702	-455
	NNY	-609	36.3	0	-732	-485
	NYN	0.4	36.3	1	-123	124
	NNN	-706	36.3	0	-830	-582

Table 4.15 (cont. on next page)

B

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
YNN	**YYY**	596	36.3	0	472	720
	YNY	83.6	36.3	0.778	-40	207
	NYY	579	36.3	0	455	703
	YYN	579	36.3	0	455	702
	NNY	-30	36.3	1	-154	93.6
	NYN	579	36.3	0	456	703
	NNN	-127	36.3	0.038	-251	-3.61
NNY	**YYY**	626	36.3	0	502	750
	YNY	114	36.3	0.103	-9.99	237
	NYY	609	36.3	0	485	733
	YYN	609	36.3	0	485	732
	YNN	30	36.3	1	-93.6	154
	NYN	609	36.3	0	486	733
	NNN	-97.2	36.3	0.323	-221	26.4
NYN	**YYY**	16.8	36.3	1	-107	140
	YNY	-496	36.3	0	-619	-372
	NYY	-0.2	36.3	1	-124	123
	YYN	-0.4	36.3	1	-124	123
	YNN	-579	36.3	0	-703	-456
	NNY	-609	36.3	0	-733	-486
	NNN	-706	36.3	0	-830	-583
NNN	**YYY**	723	36.3	0	600	847
	YNY	211	36.3	0	87.2	334
	NYY	706	36.3	0	583	830
	YYN	706	36.3	0	582	830
	YNN	127	36.3	0.038	3.61	251
	NNY	97.2	36.3	0.323	-26.4	221
	NYN	706	36.3	0	583	830
*. The mean difference is significant at the 0.05 level.						

Table 4.15. Bonferroni's post hoc test for the effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. Bonferroni's post hoc test was conducted to compare difference between each treatment condition. P (Sig): significance level; df: degrees of freedom.

	Sum of Squares	df	Mean Square	F	P
Between Groups	13600	4	3400	72.8	
Within Groups	888	19	46.8		
Total	14500	23			

Table 4.16. One-way ANOVA test for the effect of Lipid M2 on H1N1 Binding to MDCK cells. F and P (Sig.) value were conducted through one-way ANOVA. Sig.: significance level; df: degrees of freedom.

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
					Lower Bound	Upper Bound
YNN	**NYN**	-9.57	4.14	0.322	-22.7	3.57
	NNY	11.6	4.41	0.169	-2.46	25.6
	NYY	29.7	4.14	0	16.6	42.9
	NNN	-45.4	4.41	0	-59.4	-31.4
NYN	**YNN**	9.57	4.14	0.322	-3.57	22.7
	NNY	21.1	4.59	0.002	6.57	35.7
	NYY	39.3	4.32	0	25.6	53
	NNN	-35.9	4.59	0	-50.4	-21.3
NNY	**YNN**	-11.6	4.41	0.169	-25.6	2.46
	NYN	-21.1	4.59	0.002	-35.7	-6.57
	NYY	18.2	4.59	0.008	3.63	32.7
	NNN	-57	4.83	0	-72.3	-41.6
NYY	**YNN**	-29.7	4.14	0	-42.9	-16.6
	NYN	-39.3	4.32	0	-53	-25.6
	NNY	-18.2	4.59	0.008	-32.7	-3.63
	NNN	-75.2	4.59	0	-89.7	-60.6
NNN	**YNN**	45.4	4.41	0	31.4	59.4
	NYN	35.9	4.59	0	21.3	50.4
	NNY	57	4.83	0	41.6	72.3
	NYY	75.2	4.59	0	60.6	89.7
*. The mean difference is significant at the 0.05 level.						

Table 4.17. Bonferroni's post hoc test for the effect of lipid M2 treatment conditions of MDCK Cells on H1N1 infectivity. Bonferroni's post hoc test was conducted to compare difference between each treatment condition. P (Sig): significance level; df: degrees of freedom.

CHAPTER 5: DISCUSSION

Our investigations of natural product inhibitors of respiratory viral infection required the design of a screening assay to rapidly and precisely measure antiviral inhibitory activity *in vitro*. For this purpose, indirect and direct FFU blocking assays were designed and performed using some modifications of a previously developed assay (J. K. Johnson, Schmidt, Gelberg, & Kuhlenschmidt, 2004). In the indirect blocking assay, inhibitors were added to host cells at different times (before, during and after virus adsorption) using eight different combinations. By using these different combinations, we are able to examine which step during the virus-host cell infection cycle the inhibitor blocked.

The results of our initial screen, using HMO (Table 4.1), show that 3-SL and 3-FL may be candidate inhibitors for H1N1 infection. It is generally known that the infection of many bacteria and viruses occurs by binding to particular glycans on the surface of epithelial cells. Some of these glycan-binding determinants are also part of HMO, suggesting that HMO may serve as soluble receptor analogs, block pathogen adhesion, and protect the breastfed infant against certain microbial infections (Kobata, 2010). Suzuki et al. pointed out the attachment of influenza virus to epithelial cells occurs via a binding domain on the HA which recognizes SA containing glycans on the surface of epithelial cell (Y. Suzuki, 2005). This may explain why SA, albeit it at relatively high concentration, can inhibit H1N1 infectivity. Thus, it is likely 3'-SL and 3'-FL behave as soluble receptors for H1N1 attachment. Receptors on host cells and

these oligosaccharides compete for attachment by the viral binding protein (HA). However, considering the IC_{50} of 3-SL and 3-FL(~ 5mg/ml), the use of these HMO may not be economically feasible, especially as routine additives to infant formula. This result is not particularly surprising, since the affinity of soluble small oligosaccharides or monosaccharides for the combining site in carbohydrate binding proteins is generally low, typically ranging from micro- to millimolar (W. I. Weis & Drickamer, 1996). It is quite possible one could compensate for these weak monovalent interactions by coupling HMO to protein or lipid adducts to form neoglycoconjugates which more closely mimic the complex carbohydrate structures found on host cell surfaces that are exploited by many microbial pathogens. This is typically achieved by assembling individual oligosaccharides into multimers, to allow multivalent carbohydrate binding proteins to simultaneously engage many individual oligosaccharides analogs (W. I. Weis & Drickamer, 1996; William I, 1997). This "cooperative multiplicity" of low affinity combining sites has the statistical effect of significantly increasing the avidity of carbohydrate binding proteins for their intend targets (Mulvey, Kitov, Marcato, Bundle, & Armstrong, 2001). While multimerization of oligosaccharides can overcome these weak monovalent interactions, it presents another problem: these synthetic multimers are no longer natural molecules. Such derivatives would be considered drugs rather than natural products and thus may not be acceptable as a routine additive to human infant formula. The safety of such new drugswould need to be rigorously tested before they could be added into infant food and may never be acceptable to consumers.

Following completion of the HMO screening assays, we began to search for other potential nutraceutical or natural product inhibitors that may possess more potent inhibitory activity against respiratory viruses such as H1N1. For this purpose, we screened a variety of infant formulas and human milk. Among all these compounds tested, XGE, XPE infant formulas, and human milk were found have strong antiviral activity. They were able to inhibit virus infectivity at concentrations below 8mg/ml. Furthermore, compared to XGE, the antiviral activities of XPE and human milk were remarkable anddisplayed IC_{50}values of 0.98 mg/ml and 1.95 mg/ml respectively. In this regard, it is worth noting that the inhibition curve of XPE is similar to that of human milk. These results may suggest that both XPE and human milk contain similar antiviral compounds.

To characterize XPE's mechanism of inhibition, we first sought to determine if XPE acts on the virus or on the MDCK cell to block viral infection. Using the indirect blocking assay, XPE was able to at least partially block H1N1 infectivity regardless of whether it was added before, during and after, virus adsorption. This result suggestedXPEmay act on both the virus and host cells to inhibit virus infection. It is worth noting that when XPE was only added to host cells prior to virus inoculation and then removed, or after virus adsorption was complete, virus infectivity could still be reduced by 81%, and 39%, respectively. Although the most effective (>95% inhibition) combination was when XPE was present in all three stages of the virus

infection cycle (before, during, after virus adsorption), these results suggested XPE has the potential to protect as well as treat ongoing H1N1 infection.

Based on these early promising results, we began to investigate which component of XPE was responsible for the antiviral activity. XPE is composed of a fat system and a protein system. Compared to XPE, XHE has the same protein system but no fat system and displays no antiviral inhibitory activity. Accordingly, we hypothesizedthe fat system was responsible for the antiviral ability. The fat system and the protein system of XPE were separated and tested using the direct blocking assy. The results showed organic solvent extracts possessed a stronger antiviralactivity than intact XPE, while aqueous extracts contained little or no inhibitory ability. These results confirmed our initial speculation that the active antiviral component of XPE was contained in the fat system of this infant formula.

To further characterize the viral inhibitory activity detected in organic solvent extracts of XPE, TLC was used to fractionate and analyze the organic extracts. Each separate fraction was purified and screened for itsantiviral activity by the direct blocking assay. A single band (Mid2) was found to possess almost all of the inhibitory activity of the entire organic extract. Furthermore, the results of indirect blocking assays showed that Mid2 displayed the same inhibitory characteristicsof intact XPE, which can inhibit virus infectivity when added before virus inoculation, and then removed or added after virus inoculation. These results indicate Mid2 is the active lipid, which is

primarily responsible for the antiviral activity of XPE.

In an effort to identity the chemical nature of the Mid2,we compared the thin layer chromatographic mobility of Mid2 and XPE lipid extracts withseveral commercial lipids and fatty acids. None of these lipid standards co-chromatographed with Mid2. Furthermore, the organic solvent extract of XGE, which has the same protein system as XPE but lacks its predigested fat component and antiviral activity, was analyzed by TLC. Although this comparison of the lipid profile of XPE and XGE clearly indicated Mid2 originated from the predigested fat component of XPE, the exact chemical identify of Mid2 lipid has yet to be determined. In addition, organic solvent extraction of human milk, using the same procedure as for XPE,demonstrated the antiviral activity of human milk was recovered in lipid fraction and contains a lipid band displaying similar TLC mobility as observed for Mid2. Whether these two lipids have similar structure has yet to be determined. Nevertheless, these results demonstrate specific milk and infant formula derived lipids possess antiviral activity.

To begin to address the mechanism by which Mid2 or milk derived lipids block virus infection, we tested whether Mid2 acts solely by blocking virus attachment to host cells or if it can also interfere with virus entry into the cell. For these experiments, the blocking assay was performed in the presence and absence of Mid2 by first allowing viral attachment at 4 C. Under this condition, virus can only attach to but not enter the cell. Following adsorption, the cells were washed free of unbound virus and then the

temperature was raised to 37 C to allow virus entry. The result of these experiments indicated Mid2 or XPE inhibited virus infectivity by approximately 50% when added during the 4 C virus attachment stage as compared to 96% inhibition when added during 37 C incubations. Addition of Mid2 only during the viral entry stage (aftervirus adsorption at 4 C, washing to remove unbound virus, and then adding Mid2 followed by warming to 37 C) resulted in approximately a 40-45% inhibition of virus infectivity. The combined inhibitory effect of Mid2 on both virus attachment and entry is approximately 90-95% which agrees closely with the amount of inhibition (~96%) seen when either Mid2 or XPE is added at 37 C at the beginning of virus infectivity (when virus, cells and inhibitor or added at the same time at 37 C). These results strongly suggest Mid2 blocks not only virus attachment but alsovirus entry.

Previously reported studies also have indicated human milk lipids can inhibit the infectivity of enveloped viruses. Halldor et al. concluded that antiviral activity was found in storage milk (Thormar, Isaacs, Brown, Barshatzky, & Pessolano, 1987). Isaacs et al. found that fresh milk treated with antibody to secretory IgA did not inhibit virus but became antiviral effect after 1h in the stomachs of suckling infants (C. E. Isaacs, Thormar, & Pessolano, 1986). Subsequent studiesfrom this same group indicated the occurrence of antiviral activity depended on the release of free fatty acids by milk lipases (Thormar, et al, 1994). These authors concluded that milk-derived antiviral fatty acids were found to affect the viral envelope, causing leakage

and, at higher concentrations, a complete disintegration of the envelope and the viral particles and suggested that lipids commonly found in natural products could possibly be used as antiviral agents against enveloped viruses.Sakar et al also observed the cream fraction of human milk was able to effect degradation of the viral envelope of mammary tumor virus (N. H. Sarkar, Charney, Dion, & Moore, 1973). The lowest concentration of Mid2 causing >90% inhibition of virus activity in our studies was approximately 50 μM (0.015 mg/ml XPE equivalents assuming 50% the lipid in XPE is Mid2 and assuming it is a fatty acid with an average molecular weight of ~300. Although, this concentration is far below (nearly 3 orders of magnitude) the concentration (2-20 mM) used in the studies mentioned above (Thormar, et al, 1994), where a direct effect on virus envelop integrity was observed, we can not rule out the possibility of a direct effect of lipid Mid2 on H1N1 envelope structure. Although the exact mechanism of action of lipid Mid2 is not clear, andeven though it has been suggested that the fatty acids or their monoesters might be incorporated into the lipid membrane, causing disruption of viral envelopes (Cullis & Hope, 1978), it is also possible, given the relative low concentration of lipid Mid2 necessary for antiviral activity, that other more complex mechanisms, such as interference with signal transduction, may be operative in the case of Mid2.

In sum, we have shown that a predigested lipid, which may also exist in human milk, strongly inhibits H1N1 infectivity in vitro. Continued purification of the active lipid from XPE infant formula and human milk, followed by further chemical

characterization studies, as well as determination of its exact mechanism of inhibition of H1N1 infectivity, may ultimately provide an avenue for its use as a natural therapeutic or prophylactic nutriceutical to reduce respiratory virus infection in infants.

CHAPTER 6: CONCLUSION

This study was undertaken to experimentally address the hypothesis that bioactive natural products, such as those found in commercial infant formula or human milk, can function as antiviral nutriceuticals for the treatment or prevention of respiratory disease in infants and young children. The hypothesis was tested by screening the antiviral effect of some known antimicrobial compounds, such as oligosaccharides and lipids present in human milk and certain infant formulas, for their ability to inhibit respiratory virus infectivity *in vitro*. The results of the initial screening experiments demonstrated certain oligosaccharides, 3'-SL and 3'-FL, found in human milk and a lipid fraction extracted from selected commercial infant formula, possess antiviral activity. Although 3'-SL and 3'-FL possess antiviral activity, a comparison of their IC_{50} values reveals effective inhibition requires mg/ml concentration, a range that may not be economically feasible for routine inclusion as antiviral nutriceutical compounds in infant formula.

On the other hand, a remarkable antiviral activity was found in one of the infant formula (XPE) tested. Further investigation revealed a lipid fraction (Mid2) is primarily responsible for the antiviral ability of XPE. The purified Mid2 displays greater anti-viral specific activity than crude XPE. Furthermore, an appealing characteristic of mid2 is that when MDCK cells are pretreated with Mid2 followed by removal of Mid2 before virus adsorption, or if Mid2 is added to cells after virus adsorption, virus infectivity is still inhibited. While the complete chemical

characterization of Mid2 is not completed, we suspect this lipid is fatty acid derived froma predigested fat component present in XPE. We also believe this or similar lipid is present in human milk based our preliminary fractionation results. Further analysis such as Fast Atom Bombardment Mass Spectrometry (FAB-MS) and NMR is underway and should help to identify the exact chemical structure of lipid Mid2. The exact mechanism of Mid2 antiviral activity also is not clear. It is possible lipid Mid2 could be incorporated into the host cell membrane and cause destabilization of the bilayer via a detergent-like effect. Such a mechanism might also lead to the disruption of the integrityor the viral envelop. Although we have not systematically tested this possibility, the concentrations of lipid Mid2 capable of inhibiting >90% of virus infectivity caused no obvious CPE or damage to host cell monolayers. Accordingly, a likely alternative hypothesis for the mechanism of action of lipid Mid2 is that it specifically binds to the H1N1 envelope and competes with virus-host cell binding receptors. This virus binding mechanism coupled with uptake of exogenous lipid Mid2 by host cells could explain why lipid Mid2 is active not only during virus-host cell binding and entry but also during virus replication/maturation.

In summary, the results of this study illustrate the potential value of selected natural products, such as human milk-derived lipids and oligosaccharides, for use as nutriceuticals in prevention or therapy of viral respiratory diseases in infants and young children. In particular, the use of such bioactive nutriceutical compounds, such as lipid Mid2, may help augment or overcome the limitations of current antivirals, the

unavailability of effective vaccines for respiratory viral diseases, and the immaturity of infant immune system.

REFERENCES

Adcock, P. M., Stout, G. G., Hauck, M. A., & Marshall, G. S. (1997). Effect of rapid viral diagnosis on the management of children hospitalized with lower respiratory tract infection. *Pediatr Infect Dis J, 16*(9), 842-846.

Andres, A., Donovan, S. M., Kuhlenschmidt, T. B., & Kuhlenschmidt, M. S. (2007). Isoflavones at concentrations present in soy infant formula inhibit rotavirus infection in vitro. *J Nutr, 137*(9), 2068-2073.

Becker, Y. (2006). Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergyâ€"a review. *Virus Genes, 33*(2), 235-252. doi:10.1007/s11262-006-0064-x

Behera, A. K., Matsuse, H., Kumar, M., Kong, X., Lockey, R. F., & Mohapatra, S. S. (2001). Blocking intercellular adhesion molecule-1 on human epithelial cells decreases respiratory syncytial virus infection. *Biochemical and Biophysical Research Communications, 280*(1), 188-195.

Bergner, D. W., Kuhlenschmidt, T. B., Hanafin, W. P., Firkins, L. D., & Kuhlenschmidt, M. S. (2011). Inhibition of rotavirus infectivity by a

neoglycolipid Receptor Mimetic. *Nutrients, 3*(2), 228-244. doi:10.3390/nu3020228

Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., & Zako, M. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annual Review of Biochemistry, 68*(1), 729-777. doi:10.1146/annurev.biochem.68.1.729

Black, R. E., Morris, S. S., & Bryce, J. (2003). Where and why are 10 million children dying every year? [Abstract]. *The Lancet, 361*(9376) 2226-2234.

Bode, L. (2009). Human milk oligosaccharides: Prebiotics and beyond. *Nutrition Reviews, 67,* S183-S191. doi:10.1111/j.1753-4887.2009.00239.x

Brady, M. T., Sears, S. D., Pacini, D. L., Samorodin, R., DePamphilis, J., Oakes, M., . . . Clements, M. L. (1990). Safety and prophylactic efficacy of low-dose rimantadine in adults during an influenza A epidemic. *Antimicrob Agents Chemother, 34*(9), 1633-1636.

Brandenburg, A. H., Kleinjan, A., van het Land, B., Moll, H. A., Timmerman, H. H., de Swart, R. L., . . . Osterhaus, A. D. M. E. (2000). Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. *Journal of Medical Virology, 62*(2), 267-277. doi:10.1002/1096-9071(200010)62:2<267::AID-JMV20>3.0.CO;2-8

Brandenburg, A. H., van Beek, R., Moll, H. A., Osterhaus, A. D. M. E., & Claas, E. C. J. (2000). G protein variation in respiratory syncytial virus group A does not correlate with clinical severity. *Journal of Clinical Microbiology, 38*(10), 3849-3852.

Brown, G., Jeffree, C. E., McDonald, T., McL. Rixon, H. W., Aitken, J. D., & Sugrue, R. J. (2004). Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. *Virology, 327*(2), 175-185.

Castro, M., Schweiger, T., Yin-DeClue, H., Ramkumar, T. P., Christie, C., Zheng, J., . . . Bacharier, L. B. (2008). Cytokine response after severe respiratory syncytial virus bronchiolitis in early life [Abstract]. *The Journal of Allergy and Clinical Immunology, 122*(4) 726-733.e3.

Chen, R. L., & Lander, A. D. (2001). Mechanisms underlying preferential assembly of heparan sulfate on glypican-1. *Journal of Biological Chemistry, 276*(10), 7507-7517. doi:10.1074/jbc.M008283200

Chirico, G., Marzollo, R., Cortinovis, S., Fonte, C., & Gasparoni, A. (2008). Antiinfective properties of human milk. *The Journal of Nutrition, 138*(9), 1801S-1806S.

Clover, R. D., Crawford, S. A., Abell, T. D., Ramsey, C. N., Glezen, W. P., & Couch, R. B. (1986). Effectiveness of rimantadine prophylaxis of children within families. *Am J Dis Child, 140*(7), 706-709.

Colamussi, M. L., White, M. R., Crouch, E., & Hartshorn, K. L. (1999). Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. *Blood, 93*(7), 2395-2403.

Collins, P. L., & Graham, B. S. (2008). Viral and host factors in human respiratory syncytial virus pathogenesis. *The Journal of Virology, 82*(5), 2040-2055. doi:10.1128/JVI.01625-07

Cooper, A. C., Banasiak, N. C., & Allen, P. J. (2003). Management and prevention strategies for respiratory syncytial virus (RSV) bronchiolitis in infants and young children: A review of evidence-based practice interventions. *Pediatr Nurs, 29*(6), 452-456.

Couch, R. B. (2000). Influenza: Prospects for control. *Annals of Internal Medicine, 133*(12), 992-998.

Cullis, P. R., & Hope, M. J. (1978). Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. *Nat, 271*(5646), 672-674. doi:10.1038/271672a0

Darville, T., & Yamauchi, T. (1998). Respiratory syncytial virus. *Pediatrics in Review, 19*(2), 55-61. doi:10.1542/pir.19-2-55

DeVincenzo, J. P. (2005). Factors predicting childhood respiratory syncytial virus severity: What they indicate about pathogenesis. *The Pediatric Infectious Disease Journal, 24*(11)

Didierlaurent, A., Goulding, J., Patel, S., Snelgrove, R., Low, L., Bebien, M., . . . Hussell, T. (2008). Sustained desensitization to bacterial toll-like receptor ligands after resolution of respiratory influenza infection. *Exp, 205*(2), 323-329. doi:10.1084/jem.20070891

DIEFENBACH, H., & WINTER, H. (1958). [Clinical complications of the viral influenza of 1957 in comparison to influenza epidemics in preceding years.]. *Medizinische, 35*, 1271-1273.

Dobos, P. (1976). Use of gum tragacanth overlay, applied at room temperature, in the plaque assay of fish and other animal viruses. *Journal of Clinical Microbiology, 3*(3), 373-375.

Ebrahim, G. J. (1997). Breastmilk oligosaccharides point the way to new therapeutic strategies. *Journal of Tropical Pediatrics, 43*(1), 2-3.

Eisenhut, M. (2006). Extrapulmonary manifestations of severe respiratory syncytial virus infection â€“ a systematic review. *Critical Care, 10*(4), 1-6. doi:10.1186/cc4984

Espinosa, R., Tamez, M., & Prieto, P. (2007). Efforts to emulate human milk oligosaccharides. *Br J Nutr, 98 Suppl 1*, S74-9.

Falkler, W. A., Diwan, A. R., & Halstead, S. B. (1975). A lipid inhibitor of dengue virus in human colostrum and milk; with a note on the absence of anti-dengue secretory antibody. *Archives of Virology, 47*(1), 3-10. doi:10.1007/BF01315587

Falsey, A. R., & Walsh, E. E. (2000). Respiratory syncytial virus infection in adults. *Clinical Microbiology Reviews, 13*(3), 371-384. doi:10.1128/CMR.13.3.371-384.2000

Fieldsteel, A. H. (1974). Nonspecific antiviral substances in human milk active against arbovirus and murine leukemia virus. *Cancer Research, 34*(4), 712-715.

Figueras-Aloy, J., Carbonell-Estrany, X., Quero, J., & IRIS, S. G. (2004). Case-control study of the risk factors linked to respiratory syncytial virus infection requiring hospitalization in premature infants born at a gestational age of 33-35 weeks in spain. *Pediatr Infect Dis J, 23*(9), 815-820. doi:10.1097/01.inf.0000136869.21397.6b

Frevert, C. W., & Sannes, P. L. (2005). Matrix proteoglycans as effector molecules for epithelial cell function. *European Respiratory Review, 14*(97), 137-144. doi:10.1183/09059180.05.00009703

Galbraith, A. W., Oxford, J. S., Schild, G. C., & Watson, G. I. (1969). Protective effect of 1-adamantanamine hydrochloride on influenza A2 infections in the family environment: A controlled double-blind study. *Lancet, 2*(7629), 1026-1028. doi:10.1016/S0140-6736(69)90639-4

Gamberucci, A., Fulceri, R., & Benedetti, A. (1997). Inhibition of storen-dependent capacitative Ca2+ influx by unsaturated fatty acids. *Cell Calcium, 21*(5), 375-385.

Garofalo, R. P., Patti, J., Hintz, K. A., Hill, V., Ogra, P. L., & Welliver, R. C. (2001). Macrophage inflammatory protein-1α (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. *Journal of Infectious Diseases, 184*(4), 393-399. doi:10.1086/322788

Ge, S., & Wang, Z. (2011). An overview of influenza A virus receptors. *Critical Reviews in Microbiology, 37*(2), 157-165. doi:10.3109/1040841X.2010.536523

Glezen, W. P., Loda, F. A., Clyde, J.,Wallace A., Senior, R. J., Sheaffer, C. I., Conley, W. G., & Denny, F. W. (1971). Epidemiologic patterns of acute lower respiratory disease of children in a pediatric group practice. *The Journal of Pediatrics, 78*(3), 397-406. doi:DOI: 10.1016/S0022-3476(71)80218-4

Graham, B. S., Johnson, T. R., & Peebles, R. S. (2000). Immune-mediated disease pathogenesis in respiratory syncytial virus infection. *Immunopharmacology, 48*(3), 237-247.

Greenberg, H. B., & Piedra, P. A. (2004). Immunization against viral respiratory disease: A review. *The Pediatric Infectious Disease Journal, 23*(11)

Groothuis, J. R., Hoopes, J. M., & Jessie, V. G. (2011). Prevention of serious respiratory syncytial virus-related illness. I: Disease pathogenesis and early attempts at prevention. *Adv Ther, 28*(2), 91-109. doi:10.1007/s12325-010-0100-z

Groskreutz, D. J., Monick, M. M., Powers, L. S., Yarovinsky, T. O., Look, D. C., & Hunninghake, G. W. (2006). Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. *The Journal of Immunology, 176*(3), 1733-1740.

Hacking, D., & Hull, J. (2002). Respiratory syncytial virus--viral biology and the host response. *Journal of Infection, 45*(1), 18-24.

Hall, C. B. (2001). Respiratory syncytial virus and parainfluenza virus. *New England Journal of Medicine, 344*(25), 1917-1928.

Hall, C. B., Weinberg, G. A., Iwane, M. K., Blumkin, A. K., Edwards, K. M., Staat, M. A., . . . Szilagyi, P. (2009). The burden of respiratory syncytial virus infection in young children. *N Engl J Med, 360*(6), 588-598. doi:10.1056/NEJMoa0804877

Hall, C. (1999). Respiratory syncytial virus: A continuing culprit and conundrum. *J Pediatr, 135*(2), 2-7.

Hallak, L. K., Collins, P. L., Knudson, W., & Peeples, M. E. (2000). Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. *Virology, 271*(2), 264-275.

Hamosh, M. (2001). Bioactive factors in human milk [Abstract]. *Pediatric Clinics of North America, 48*(1) 69-86.

Harper, S. A., Bradley, J. S., Englund, J. A., File, T. M., Gravenstein, S., Hayden, F. G., . . . Zimmerman, R. K. (2009). Seasonal influenza in adults and Children—Diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: Clinical practice guidelines of the infectious diseases society of america. *Clinical Infectious Diseases, 48*(8), 1003-1032. doi:10.1086/598513

Haynes, L. M., Moore, D. D., Kurt-Jones, E. A., Finberg, R. W., Anderson, L. J., & Tripp, R. A. (2001). Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *The Journal of Virology, 75*(22), 10730-10737. doi:10.1128/JVI.75.22.10730-10737.2001

Hertz, M. I., Englund, J. A., Snover, D., Bitterman, P. B., & McGlave, P. B. (1989). Respiratory syncytial virus-induced acute lung injury in adult patients with bone marrow transplants: A clinical approach and review of the literature. *Medicine, 68*(5)

Higa, H. H., Rogers, G. N., & Paulson, J. C. (1985). Influenza virus hemagglutinins differentiate between receptor determinants bearing N-acetyl-, N-glycollyl-, and N,O-diacetyineuraminic acids. *Virology, 144*(1), 279-282.

Hilmarsson, H., Traustason, B. S., Kristmundsdóttir, T., & Thormar, H. (2007). Virucidal activities of medium- and long-chain fatty alcohols and lipids against respiratory syncytial virus and parainfluenza virus type 2: Comparison at different pH levels. *Archives of Virology, 152*(12), 2225-2236. doi:10.1007/s00705-007-1063-5

Hosea Blewett, H. J., Cicalo, M. C., Holland, C. D., & Field, C. J. (2008). The immunological components of human milk. In Steve L. Taylor (Ed.), *Advances in food and nutrition research* (pp. 45-80) Academic Press.

Hull, J., Thomson, A., & Kwiatkowski, D. (2000). Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. *Thorax, 55*(12), 1023-1027.

Isaacs, C. E., Kashyap, S., Heird, W. C., & Thormar, H. (1990). Antiviral and antibacterial lipids in human milk and infant formula feeds. *Archives of Disease in Childhood, 65*(8), 861-864. doi:10.1136/adc.65.8.861

Isaacs, C. E. (2011). Antimicrobial lipids in milk. In *Lipids and essential oils as antimicrobial agents* (pp. 81-97) John Wiley & Sons, Ltd. doi:10.1002/9780470976623.ch4

ISAACS, C. E., SOO KIMS, K., & THORMAR, H. (1994). Inactivation of enveloped viruses in human bodily fluids by purified lipidsa. *Annals of the New York Academy of Sciences, 724*(1), 457-464. doi:10.1111/j.1749-6632.1994.tb38947.x

Isaacs, C. E., Thormar, H., & Pessolano, T. (1986). Membrane-disruptive effect of human milk: Inactivation of enveloped viruses. *Journal of Infectious Diseases, 154*(6), 966-971. doi:10.1093/infdis/154.6.966

Ito, T., Couceiro, J. N. S. S., Kelm, S., Baum, L. G., Krauss, S., Castrucci, M. R., . . . Kawaoka, Y. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *Journal of Virology, 72*(9), 7367-7373.

Ito, T., & Kawaoka, Y. (2000). Host-range barrier of influenza A viruses. *Veterinary Microbiology, 74*(1-2), 71-75.

Ito, T., Suzuki, Y., Mitnaul, L., Vines, A., Kida, H., & Kawaoka, Y. (1997). Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. *Virology, 227*(2), 493-499.

Jeffree, C. E., Brown, G., Aitken, J., Su-Yin, D., Tan, B., & Sugrue, R. J. (2007). Ultrastructural analysis of the interaction between F-actin and respiratory syncytial virus during virus assembly. *Virology, 369*(2), 309-323.

Jeffree, C. E., Rixon, H. W. M. L., Brown, G., Aitken, J., & Sugrue, R. J. (2003). Distribution of the attachment (G) glycoprotein and GM1 within the envelope of

mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology, 306*(2), 254-267.

Jensen, R. G. (1996). The lipids in human milk. *Progress in Lipid Research, 35*(1), 53-92.

Johnson, J. K., Schmidt, J., Gelberg, H. B., & Kuhlenschmidt, M. S. (2004). Microbial adhesion of cryptosporidium parvum sporozoites: Purification of an inhibitory lipid from bovine mucosa. *J Parasitol, 90*(5), 980-990. doi:10.1645/GE-231R

Johnson, J. E., Gonzales, R. A., Olson, S. J., Wright, P. F., & Graham, B. S. (2006). The histopathology of fatal untreated human respiratory syncytial virus infection. *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc, 20*(1), 108-119.

Kabara, J. J. (1980). Lipids as host-resistance factors of human milk. *Nutrition Reviews, 38*(2), 65-73. doi:10.1111/j.1753-4887.1980.tb05843.x

Kabara, J. J., Swieczkowski, D. M., Conley, A. J., & Truant, J. P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents and Chemotherapy, 2*(1), 23-28. doi:10.1128/AAC.

Kallal, L. E., & Lukacs, N. W. (2008). The role of chemokines in virus-associated asthma exacerbations. *Curr Allergy Asthma Rep, 8*(5), 443-450.

Klugman, K. P., & Madhi, S. A. (2007). Pneumococcal vaccines and flu preparedness. *Science, 316*(5821), 49-50. doi:10.1126/science.316.5821.49c

Kneyber, M. C., Brandenburg, A. H., de Groot, R., Joosten, K. F., Rothbarth, P. H., Ott, A., & Moll, H. A. (1998). Risk factors for respiratory syncytial virus associated apnoea. *Eur J Pediatr, 157*(4), 331-335.

Knight, V., Fedson, D., Baldini, J., Douglas, R. G., & Couch, R. B. (1970). Amantadine therapy of epidemic influenza a(2) (hong kong). *Infect Immun, 1*(2), 200-204.

Kobasa, D., Takada, A., Shinya, K., Hatta, M., Halfmann, P., Theriault, S., . . . Kawaoka, Y. (2004). Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature, 431*(7009), 703-707.

KOBATA, A. (2010). Structures and application of oligosaccharides in human milk. *Proceedings of the Japan Academy, Series B, 86*(7), 731-747.

Kohn, A., Gitelman, J., & Inbar, M. (1980). Unsaturated free fatty acids inactivate animal enveloped viruses. *Archives of Virology, 66*(4), 301-307. doi:10.1007/BF01320626

Korppi, M., Piippo-Savolainen, E., Korhonen, K., & Remes, S. (2004). Respiratory morbidity 20 years after RSV infection in infancy. *Pediatr Pulmonol, 38*(2), 155-160. doi:10.1002/ppul.20058

Kunz, C., Rudloff, S., Baier, W., Klein, N., & Strobel, S. (2000). OLIGOSACCHARIDES IN HUMAN MILK: Structural, functional, and metabolic aspects. *Annual Review of Nutrition, 20*(1), 699-722. doi:10.1146/annurev.nutr.20.1.699

Kunz, C., & Rudloff, S. (1993). Biological functions of oligosaccharides in human milk. *Acta Pædiatrica, 82*(12), 903-912. doi:10.1111/j.1651-2227.1993.tb12597.x

Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., . . . Finberg, R. W. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol, 1*(5), 398-401. doi:10.1038/80833

Langley, G. F., & Anderson, L. J. (2011). Epidemiology and prevention of respiratory syncytial virus infections among infants and young children. *Pediatr Infect Dis J,* doi:10.1097/INF.0b013e3182184ae7

Leader, S., & Kohlhase, K. (2002). Respiratory syncytial virus-coded pediatric hospitalizations, 1997 to 1999. *Pediatr Infect Dis J, 21*(7), 629-632. doi:10.1097/01.inf.0000019891.59210.1c

Lee, N., Hui, D., Wu, A., Chan, P., Cameron, P., Joynt, G. M., . . . Sung, J. J. (2003). A major outbreak of severe acute respiratory syndrome in hong kong. *NEJM, 348*(20), 1986-1994. doi:10.1056/NEJMoa030685

Li, Q., Estes, J. D., Schlievert, P. M., Duan, L., Brosnahan, A. J., Southern, P. J., . . . Haase, A. T. (2009). Glycerol monolaurate prevents mucosal SIV transmission. *Nature, 458*(7241), 1034-1038.

Little, J. W., Hall, W. J., Douglas, R. G., Hyde, R. W., & Speers, D. M. (1976). Amantadine effect on peripheral airways abnormalities in influenza. A study in 15 students with natural influenza A infection. *Ann Intern Med, 85*(2), 177-182.

Lo, M. S., Brazas, R. M., & Holtzman, M. J. (2005). Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. *J Virol, 79*(14), 9315-9319. doi:10.1128/JVI.79.14.9315-9319.2005

Longini, I. M., Koopman, J. S., Monto, A. S., & Fox, J. P. (1982). Estimating household and community transmission parameters for influenza. *Am J Epidemiol, 115*(5), 736-751.

Low, D. (2008). Reducing antibiotic use in influenza: Challenges and rewards. *Clinical Microbiology and Infection, 14*(4), 298-306. doi:10.1111/j.1469-0691.2007.01910.x

Ma, W., Lager, K. M., Vincent, A. L., Janke, B. H., Gramer, M. R., & Richt, J. A. (2009). The role of swine in the generation of novel influenza viruses. *Zoonoses and Public Health, 56*(6-7), 326-337. doi:10.1111/j.1863-2378.2008.01217.x

Malhotra, R., Ward, M., Bright, H., Priest, R., Foster, M., Hurle, M., . . . Bird, M. (2003). Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. *Microbes and Infection, 5*(2), 123-133.

MARTINEZ, F. D. (2003). Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. *The Pediatric Infectious Disease Journal, 22*(2)

Mart ńez, I., & Melero, J. A. (2000). Binding of human respiratory syncytial virus to cells: Implication of sulfated cell surface proteoglycans. *Journal of General Virology, 81*(11), 2715-2722.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., . . . Kawaoka, Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *The Journal of Virology, 74*(18), 8502-8512. doi:10.1128/JVI.74.18.8502-8512.2000

McCullers, J. A., Iverson, A. R., McKeon, R., & Murray, P. J. (2008). The platelet activating factor receptor is not required for exacerbation of bacterial pneumonia following influenza. *Scand J Infect Dis, 40*(1), 11-17. doi:10.1080/00365540701477568

McNamara, P. S., & Smyth, R. L. (2002). The pathogenesis of respiratory syncytial virus disease in childhood. *British Medical Bulletin, 61*(1), 13-28. doi:10.1093/bmb/61.1.13

McNamee, L. A., & Harmsen, A. G. (2006). Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary streptococcus pneumoniae infection. *Inf Immun, 74*(12), 6707-6721. doi:10.1128/IAI.00789-06

McVEAGH, P., & MILLER, J. B. (1997). Human milk oligosaccharides: Only the breast. *Journal of Paediatrics and Child Health, 33*(4), 281-286. doi:10.1111/j.1440-1754.1997.tb01601.x

Miller, S. (2010). A community health concern: Respiratory syncytial virus and children [Abstract]. *Journal of Pediatric Nursing, 25*(6) 551-554.

Mizgerd, J. P. (2006). Lung Infectionâ€"A public health priority. *PLoS Med, 3*(2), e76.

Molinari, N. M., Ortega-Sanchez, I., Messonnier, M. L., Thompson, W. W., Wortley, P. M., Weintraub, E., & Bridges, C. B. (2007). The annual impact of seasonal influenza in the US: Measuring disease burden and costs. *Vaccine, 25*(27), 5086-5096.

Monto, A. S., & Sullivan, K. M. (1993). Acute respiratory illness in the community. frequency of illness and the agents involved. *Epidem Inf, 110*(1), 145-160.

Monto, A. S. (2002). Epidemiology of viral respiratory infections. *The American Journal of Medicine, 112*(6, Supplement 1), 4-12. doi:DOI: 10.1016/S0002-9343(01)01058-0

Morrow, A. L., & Rangel, J. M. (2004). Human milk protection against infectious diarrhea: Implications for prevention and clinical care. *Seminars in Pediatric Infectious Diseases, 15*(4), 221-228.

Morrow, A. L., Ruiz-Palacios, G. M., Jiang, X., & Newburg, D. S. (2005). Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *The Journal of Nutrition, 135*(5), 1304-1307.

Mullins, J. A., Erdman, D. D., Weinberg, G. A., Edwards, K., Hall, C. B., Walker, F. J., . . . Anderson, L. J. (2004). Human metapneumovirus infection among children hospitalized with acute respiratory illness. *Emerg Inf Dis, 10*(4), 700-705.

Mulvey, G., Kitov, P. I., Marcato, P., Bundle, D. R., & Armstrong, G. D. (2001). Glycan mimicry as a basis for novel anti-infective drugs. *Biochimie, 83*(8), 841-847. doi:DOI: 10.1016/S0300-9084(01)01291-3

Murawski, M. R., Bowen, G. N., Cerny, A. M., Anderson, L. J., Haynes, L. M., Tripp, R. A., . . . Finberg, R. W. (2009). Respiratory syncytial virus activates innate immunity through toll-like receptor 2. *J Virol, 83*(3), 1492-1500. doi:10.1128/JVI.00671-08

N.M., C. (2008). New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. *Progress in Lipid Research, 47*(1), 50-61.

Nelson, M. I., & Holmes, E. C. (2007). The evolution of epidemic influenza. *Nature Reviews. Genetics, 8*(3), 196-205.

Newburg, D. (1996). Oligosaccharides and glycoconjugates in human milk: Their role in host defense. *Journal of Mammary Gland Biology and Neoplasia, 1*(3), 271-283. doi:10.1007/BF02018080

Nicholson, K. G., McNally, T., Silverman, M., Simons, P., Stockton, J. D., & Zambon, M. C. (2006). Rates of hospitalisation for influenza, respiratory syncytial virus and human metapneumovirus among infants and young children. *Vaccine, 24*(1), 102-108.

Ninonuevo, M. R., Park, Y., Yin, H., Zhang, J., Ward, R. E., Clowers, B. H., . . . Lebrilla, C. B. (2006). A strategy for annotating the human milk glycome. *Journal of Agricultural and Food Chemistry, 54*(20), 7471-7480. doi:10.1021/jf0615810

O'Donoghue, J. M., Ray, C. G., Terry, D. W., & Beaty, H. N. (1973). Prevention of nosocomial influenza infection with amantadine. *AM J EPID, 97*(4), 276-282.

Olszewska, W., Zambon, M., & Openshaw, P. J. M. (2002). Development of vaccines against common colds. *British Medical Bulletin, 62*(1), 99-111. doi:10.1093/bmb/62.1.99

Ordway, R. W., Singer, J. J., & Walsh Jr, J. V. (1991). Direct regulation of ion channels by fatty acids. *Trends in Neurosciences, 14*(3), 96-100.

Oshansky, C. M., Zhang, W., Moore, E., & Tripp, R. A. (2009). The host response and molecular pathogenesis associated with respiratory syncytial virus infection. *Future Microbiology, 4*(3), 279-297. doi:10.2217/fmb.09.1

Pasare, C., & Medzhitov, R. (2004). Toll-like receptors: Linking innate and adaptive immunity. *Microbes Infect, 6*(15), 1382-1387. doi:10.1016/j.micinf.2004.08.018

Patriarca, P. A. (1999). New options for prevention and control of influenza. *Journal of American Medical Association, 282*(1), 75-77. doi:10.1001/jama.282.1.75

Pavia, A. T. (2011). Viral infections of the lower respiratory tract: Old viruses, new viruses, and the role of diagnosis. *Clinical Infectious Diseases, 52*(suppl 4), S284-S289. doi:10.1093/cid/cir043

Peltola, V. T., Murti, K. G., & McCullers, J. A. (2005). Influenza virus neuraminidase contributes to secondary bacterial pneumonia. *Journal of Infectious Diseases, 192*(2), 249-257. doi:10.1086/430954

Peret, T. C., Hall, C. B., Schnabel, K. C., Golub, J. A., & Anderson, L. J. (1998). Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *Journal of General Virology, 79*(9), 2221-2229.

Plotkowski, M. C., Puchelle, E., Beck, G., Jacquot, J., & Hannoun, C. (1986). Adherence of type I streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis, 134*(5), 1040-1044.

Reuman, P. D., Bernstein, D. I., Keefer, M. C., Young, E. C., Sherwood, J. R., & Schiff, G. M. (1989). Efficacy and safety of low dosage amantadine hydrochloride as prophylaxis for influenza A. *Antiviral Res, 11*(1), 27-40.

Robert C., W. (1998). Respiratory syncytial virus immunoglobulin and monoclonal antibodies in the prevention and treatment of respiratory syncytial virus infection. *Seminars in Perinatology, 22*(1), 87-95. doi:10.1016/S0146-0005(98)80010-4

Rogers, G. N., & Paulson, J. C. (1983). Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology, 127*(2), 361-373.

Rohrer, L., Winterhalter, K. H., Eckert, J., & Kohler, P. (1986). Killing of giardia lamblia by human milk is mediated by unsaturated fatty acids. *Antimicrobial Agents and Chemotherapy, 30*(2), 254-257. doi:10.1128/AAC.

Rolsma, M. D., Kuhlenschmidt, T. B., Gelberg, H. B., & Kuhlenschmidt, M. S. (1998). Structure and function of a ganglioside receptor for porcine rotavirus. *J Virol, 72*(11), 9079-9091.

Rothberg, M. B., Haessler, S. D., & Brown, R. B. (2008). Complications of viral influenza. *Med, 121*(4), 258-264. doi:10.1016/j.amjmed.2007.10.040

Rudloff, S., Pohlentz, G., Diekmann, L., Egge, H., & Kunz, C. (1996). Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. *Acta Pædiatrica, 85*(5), 598-603. doi:10.1111/j.1651-2227.1996.tb14095.x

Sánchez-Juanes, F., Alonso, J. M., Zancada, L., & Hueso, P. (2009). Glycosphingolipids from bovine milk and milk fat globule membranes: A comparative study. adhesion to enterotoxigenic escherichia coli strains. *BC, 390*(1), 31-40. doi:10.1515/BC.2009.003

Sarkar, N. H., Charney, J., Dion, A. S., & Moore, D. H. (1973). Effect of human milk on the mouse mammary tumor virus. *Can Res, 33*(3), 626-629.

Schmidt, J., & Kuhlenschmidt, M. S. (2008). Microbial adhesion of cryptosporidium parvum: Identification of a colostrum-derived inhibitory lipid. *Molecular and Biochemical Parasitology, 162*(1), 32-39.

Schneider-Schaulies, J. (2000). Cellular receptors for viruses: Links to tropism and pathogenesis. *Journal of General Virology, 81*(6), 1413-1429.

Scholtissek, C., Bürger, H., Kistner, O., & Shortridge, K. F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology, 147*(2), 287-294.

Sharon, N., & Ofek, I. (2000). Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. *Glycoconjugate Journal, 17*(7), 659-664. doi:10.1023/A:1011091029973

Shay, D. K., Holman, R. C., Roosevelt, G. E., Clarke, M. J., & Anderson, L. J. (2001). Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979-1997. *J Infect Dis, 183*(1), 16-22. doi:10.1086/317655

Shay, D. K., Holman, R. C., Newman, R. D., Liu, L. L., Stout, J. W., & Anderson, L. J. (1999). Bronchiolitis-associated hospitalizations among US children, 1980-1996. *JAMA: The Journal of the American Medical Association, 282*(15), 1440-1446. doi:10.1001/jama.282.15.1440

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., & Kawaoka, Y. (2006). Avian flu: Influenza virus receptors in the human airway. *Nature, 440*(7083), 435-436.

Sigurs, N., Gustafsson, P. M., Bjarnason, R., Lundberg, F., Schmidt, S., Sigurbergsson, F., & Kjellman, B. (2005). Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med, 171*(2), 137-141. doi:10.1164/rccm.200406-730OC

Sikkel, M. B., Quint, J. K., Mallia, P., Wedzicha, J. A., & Johnston, S. L. (2008). Respiratory syncytial virus persistence in chronic obstructive pulmonary disease. *Pediatr Infect Dis J, 27*(10), S63-S70. doi:10.1097/INF.0b013e3181684d67

Simoes, E. A. F., Tan, D. H. S., Ohlsson, A., Sales, V., & Wang, E. E. L. (2001). Respiratory syncytial virus vaccine: A systematic overview with emphasis on respiratory syncytial virus subunit vaccines. *Vaccine, 20*(5-6), 954-960.

Steinhauer, D. A., & Skehel, J. J. (2002). GENETICS OF INFLUENZA VIRUSES. *Annual Review of Genetics, 36*(1), 305-332. doi:10.1146/annurev.genet.36.052402.152757

Stevens, J., Corper, A. L., Basler, C. F., Taubenberger, J. K., Palese, P., & Wilson, I. A. (2004). Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science, 303*(5665), 1866-1870. doi:10.1126/science.1093373

Structures and application of oligosaccharides in human milk. (2010). *Proc Jpn Acad Ser B Phys Biol Sci, 86*(7), 731-747.

Susan, M. (2010). A community health concern: Respiratory syncytial virus and children. *Journal of Pediatric Nursing, 25*(6), 551-554.

Suzuki, T., Horiike, G., Yamazaki, Y., Kawabe, K., Masuda, H., Miyamoto, D., . . . Suzuki, Y. (1997). Swine influenza virus strains recognize sialylsugar chains

containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium. *FEBS Letters, 404*(2-3), 192-196.

Suzuki, Y. (2005). Sialobiology of influenza: Molecular mechanism of host range variation of influenza viruses. *Biological & Pharmaceutical Bulletin, 28*(3), 399-408.

Suzuki, Y., Ito, T., Suzuki, T., Holland, R. E., Jr., Chambers, T. M., Kiso, M., . . . Kawaoka, Y. (2000). Sialic acid species as a determinant of the host range of influenza A viruses. *The Journal of Virology, 74*(24), 11825-11831. doi:10.1128/JVI.74.24.11825-11831.2000

Tasdemir, D., Topaloglu, B., Perozzo, R., Brun, R., Oâ€™Neill, R., Carballeira, N., . . . RÃ¼edi, P. (2007). Marine natural products from the turkish sponge agelas oroides that inhibit the enoyl reductases from plasmodium falciparum, mycobacterium tuberculosis and escherichia coli. *Bioorganic & Medicinal Chemistry, 15*(21), 6834-6845.

Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L., Cox, N., Anderson, L. J., & Fukuda, K. (2003). Mortality associated with influenza and respiratory syncytial virus in the united states. *Journal of American Medical Association, 289*(2), 179-186. doi:10.1001/jama.289.2.179

Thormar, H., Isaacs, C. E., Brown, H. R., Barshatzky, M. R., & Pessolano, T. (1987). Inactivation of enveloped viruses and killing of cells by fatty acids and

monoglycerides. *Antimicrobial Agents and Chemotherapy, 31*(1), 27-31. doi:10.1128/AAC.

Togo, Y., Hornick, R. B., Felitti, V. J., Kaufman, M. L., Dawkins, A. T., Kilpe, V. E., & Claghorn, J. L. (1970). Evaluation of therapeutic efficacy of amantadine in patients with naturally occurring A2 influenza. *JAMA: The Journal of the American Medical Association, 211*(7), 1149-1156. doi:10.1001/jama.1970.03170070019004

Tregoning, J. S., & Schwarze, J. (2010). Respiratory viral infections in infants: Causes, clinical symptoms, virology, and immunology. *Clinical Microbiology Reviews, 23*(1), 74-98. doi:10.1128/CMR.00032-09

Tumpey, T. M., Garc á-Sastre, A., Taubenberger, J. K., Palese, P., Swayne, D. E., & Basler, C. F. (2004). Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. *Proceedings of the National Academy of Sciences of the United States of America, 101*(9), 3166-3171. doi:10.1073/pnas.0308391100

Urashima, T., Odaka, G., Asakuma, S., Uemura, Y., Goto, K., Senda, A., . . . Oftedal, O. T. (2009). Chemical characterization of oligosaccharides in chimpanzee, bonobo, gorilla, orangutan, and siamang milk or colostrum. *Glycobiol, 19*(5), 499-508. doi:10.1093/glycob/cwp006

Valarcher, J. F., Bourhy, H., Lavenu, A., Bourges-Abella, N., Roth, M., Andreoletti, O., . . . Schelcher, F. (2001). Persistent infection of B lymphocytes by bovine respiratory syncytial virus. *Virolog, 291*(1), 55-67. doi:10.1006/viro.2001.1083

van den Hoogen, B. G., Osterhaus, D. M., & Fouchier, R. A. (2004). Clinical impact and diagnosis of human metapneumovirus infection. *Ped-Inf, 23*(1), S25-S32. doi:10.1097/01.inf.0000108190.09824.e8

van der Sluijs, K. F., van Elden, L. J., Nijhuis, M., Schuurman, R., Florquin, S., Shimizu, T., . . . van der Poll, T. (2006). Involvement of the platelet-activating factor receptor in host defense against streptococcus pneumoniae during postinfluenza pneumonia. *American journal of physiology - lung cellular and molecular physiology, 290*(1), L194-L199. doi:10.1152/ajplung.00050.2005

van der Sluijs, K. F., van Elden, L. J., Nijhuis, M., Schuurman, R., Pater, J. M., Florquin, S., . . . van der Poll, T. (2004). IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J Immunol, 172*(12), 7603-7609.

van Schaik, S. M., Welliver, R. C., & Kimpen, J. L. L. (2000). Novel pathways in the pathogenesis of respiratory syncytial virus disease. *Pediatric Pulmonology, 30*(2), 131-138. doi:10.1002/1099-0496(200008)30:2<131::AID-PPUL8>3.0.CO;2-S

Viswanathan, K., Chandrasekaran, A., Srinivasan, A., Raman, R., Sasisekharan, V., & Sasisekharan, R. (2010). Glycans as receptors for influenza pathogenesis. *Glycoconjugate Journal, 27*(6), 561-570. doi:10.1007/s10719-010-9303-4

Wan, H., & Perez, D. R. (2006). Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. *Virology, 346*(2), 278-286.

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., & Wiley, D. C. (1988). Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature, 333*(6172), 426-431.

Weis, W. I., & Drickamer, K. (1996). Structural basis of lectin-carbohydrate recognition. *Annu Rev Biochem, 65*, 441-473. doi:10.1146/annurev.bi.65.070196.002301

Welsh, J. K., Arsenakis, M., Coelen, R. J., & May, J. T. (1979). Effect of antiviral lipids, heat, and freezing on the activity of viruses in human milk. *The Journal of Infectious Diseases, 140*(3), 322-328.

William I, W. (1997). Cell-surface carbohydrate recognition by animal and viral lectins. *Current Opinion in Structural Biology, 7*(5), 624-630. doi:10.1016/S0959-440X(97)80070-X

Williams, J. V., Harris, P. A., Tollefson, S. J., Halburnt-Rush, L. L., Pingsterhaus, J. M., Edwards, K. M., . . . Crowe, J. E. (2004). Human metapneumovirus and

lower respiratory tract disease in otherwise healthy infants and children. *NEJM, 350*(5), 443-450. doi:10.1056/NEJMoa025472

Wingfield, W. L., Pollack, D., & Grunert, R. R. (1969). Therapeutic efficacy of amantadine HCl and rimantadine HCl in naturally occurring influenza A2 respiratory illness in man. *N Engl J Med, 281*(11), 579-584. doi:10.1056/NEJM196909112811102

World Health Organization. (2005). Burden of disease project. Geneva, Switzerland.

Younkin, S. W., Betts, R. F., Roth, F. K., & Douglas, R. G. (1983). Reduction in fever and symptoms in young adults with influenza A/Brazil/78 H1N1 infection after treatment with aspirin or amantadine. *Antimicrob Agents Chemother, 23*(4), 577-582.

Zopf, D., & Roth, S. (1996). Oligosaccharide anti-infective agents [Abstract]. *The Lancet, 347*(9007) 1017-1021.